



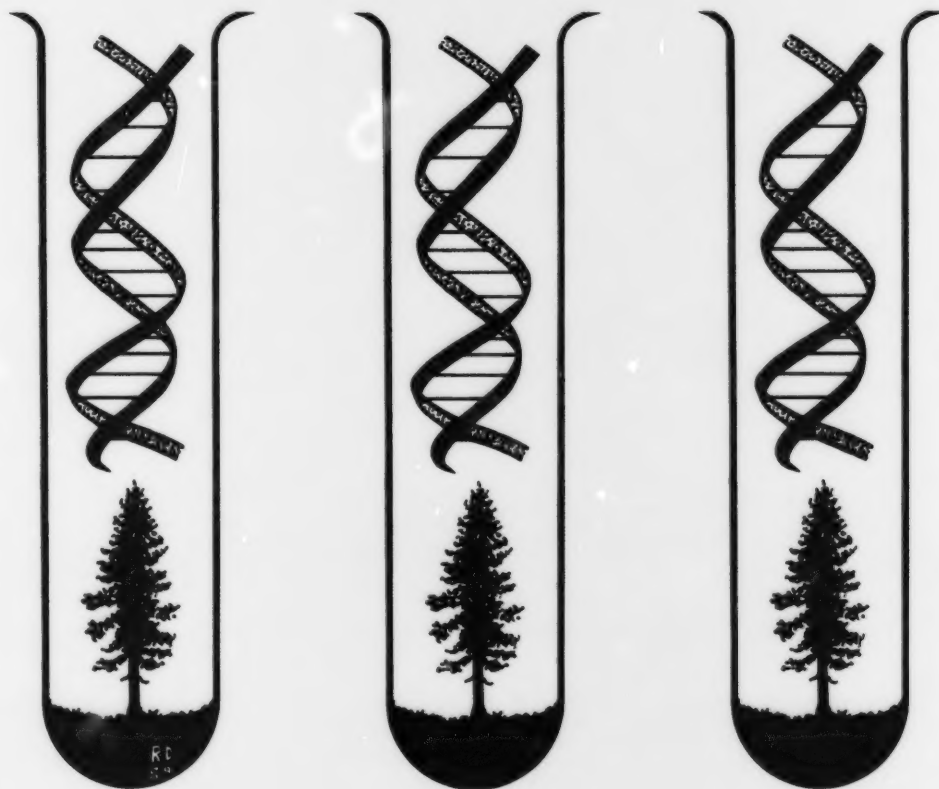
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Some protocols for cDNA library construction with conifer tissues

J.A. Pitel and W.M. Cheliak

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Petawawa National Forestry Institute



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SOME PROTOCOLS FOR cDNA LIBRARY CONSTRUCTION WITH CONIFER TISSUES

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Abstract

The Handbook presents various protocols for constructing cDNA libraries using conifer or other plant tissue as a source. Included are methods for RNA extraction that vary in terms of buffer composition, length of time for extraction, the requirement for ultracentrifugation, the degree of purification, and others. Methods for separating messenger (m) RNA from ribosomal (r)RNA, electrophoresis of mRNA, and Northern blotting are described.

For cDNA library construction, methods are presented for first and second strand synthesis, isolation of high molecular weight cDNA, electrophoresis of cDNA, ligation reactions to the vectors; extraction of insert cDNA from the vector, etc.

Also included is information that may be useful for those starting to work in this field, such as addresses for suppliers of chemicals and equipment, recipes for stock solutions, references for more detailed information, and suggestions from our experience as to which method might be successful for different tissues and species.

Résumé

Dans ce manuel, nous décrivons divers procédés permettant de constituer des banques d'ADNc à partir de tissus de conifères ou d'autres plantes. Nous donnons des méthodes d'extraction de l'ARN se distinguant par la composition du tampon, la durée de l'extraction, la nécessité de l'ultracentrifugation, le degré de purification et divers autres aspects. Nous expliquons aussi comment séparer l'ARNm (messager) de l'ARNr (ribosomique), réaliser l'électrophorèse de l'ARNm et le transfert de Northern.

En ce qui a trait à la constitution d'une banque d'ADNc, nous décrivons des méthodes de synthèse du premier et du second brin, l'isolement de l'ADNc de masse moléculaire élevée, l'électrophorèse de l'ADNc, la ligature au vecteur, l'extraction de l'ADNc inséré dans le vecteur, etc.

Enfin, nous donnons des renseignements qui seront peut-être utiles à ceux qui commencent à travailler dans le domaine. Y sont inclus notamment une liste des adresses des fournisseurs de produits chimiques et d'équipement, des recettes de solutions-mères, des sources d'information plus détaillée et des conseils quant aux méthodes qui d'après notre expérience, devraient donner les meilleurs résultats selon le type de tissu ou l'espèce.



This Handbook describes our experience in cDNA (complementary DNA) library construction of conifer seed mRNA. It is intended to be of assistance to those just starting a study in this field, or for those already involved who may want to try some of the techniques we have used with some success. Several methods were used as prescribed or modified from various sources such as journals, other Handbooks such as that of Maniatis et al. (1982), books such as "Methods in Enzymology", protocols supplied by suppliers such as Amersham and Promega, publications from chemical suppliers such as "Focus" (BRL), personal communications, and others.

Although we worked with conifers, the Handbook describes some protocols we have tried that may be suitable for other, similar tissues. Because protocols such as those for RNA extraction may vary with the species as well as for different tissues, we have presented alternative methods. For example, extraction of RNA from needle tissue was shown by Hutchison (1988) to require Triton X-100 in the extraction buffer, instead of sodium dodecyl sulfate. The latter is used for RNA extraction from seed material.

The vector chosen for cloning depends on why the library is being constructed. We have recently been working with the λ ZAP vector system from Stratagene. As the Lac Z gene is present, blue/white colour selection for recombinants is possible. Also, with this vector, one can easily obtain single- or double-stranded plasmids containing cDNA inserts. Studies that are facilitated by use of this vector include sequencing, the production of high specific activity RNA probes, *in vitro* translation studies, screening with antibody probes and nucleic acid probes, directional cloning, etc.

We also describe from the literature the many uses of cDNA cloning techniques and provide background information on RNA extraction and cloning techniques. A comprehensive reference list is included. Once the cDNA clone is isolated, it can be used to isolate the complete gene from the genomic library (eg. DeVerno et al. 1989).

2 The use of cDNA cloning techniques

2.1 Embryogenesis and germination

There have been few studies done at the molecular level with conifer species dealing with seed

embryogenesis and germination. In a couple of recent reports dealing with seed germination of jack pine (*Pinus banksiana* Lamb.) and tamarack (*Larix laricina* (Du Roi) K. Koch) we showed that, for embryos, the activity of several enzymes is low in dry and imbibed seeds but increases dramatically with radicle emergence and further growth (Pitel and Cheliak 1986, 1988a). For gametophytes, however, enzyme activity decreased during the later stages of germination of jack pine. We have completed construction of cDNA libraries at several stages of imbibition and germination of jack pine to facilitate examination of changes in gene expression during this period of growth and differentiation.

In studies with cotton (*Gossypium hirsutum*) seed embryogenesis and early germination, changes were observed in the population of six different sets of abundant mRNAs in the cotyledons as monitored by *in vivo* and *in vitro* protein synthesis (Dure 1985). For example, one set of mRNAs, say for actin, are present at all stages; a second set are from genes that are not expressed following germination; a third set of mRNAs are highly expressed only in early embryogenesis; a fourth set, representing storage protein mRNAs, are prevalent during most of embryogenesis, but then decline sharply at the end of this developmental stage; a fifth set of mRNAs become abundant in late embryogenesis but then decrease sharply after a few hours of germination; and the sixth set of mRNAs become abundant in late embryogenesis and remain abundant for several days after germination. Using nucleic acid hybridization techniques, Galau and Dure (1981) also observed changes in the less abundant mRNA classes with cotton cotyledon ontogeny. By the end of embryogenesis about 30 000 genes have been involved in producing mRNA sequences in both soybean and cotton, according to Dure (1985). At mid-embryogenesis the storage protein mRNAs are abundant. The total number of different mRNA genes for the whole plant may exceed 60 000. In later studies with cotton (eg. Chlan et al. 1986) cDNA clones that represented the three distinct groups of storage proteins of cotton seed were sequenced. In addition, using cDNA libraries, cDNA clones representing some of the mRNAs produced by late embryogenesis abundant (LEA) genes have been isolated and sequenced (Baker and Dure 1987). In a similar study with radish seeds, Laroche-Raynal et al. (1984) examined the mRNA fraction from immature, dry seed and 2 to 4 h-old seedlings. Using *in vitro* translation techniques, they observed that a subset of mRNA accumulates in late embryogenesis, remains in the dry seed (together with a few other types of mRNA), and then disappears during early germination. A number of other studies have also examined

changes in gene expression with embryogenesis and germination and the role of stored mRNA in dry seeds, often using recombinant DNA technology (eg. Suzuki and Minamikawa 1985, Koshiba et al. 1986, Reeves et al. 1986, Kreis et al. 1986, Walling et al. 1986, Bartels and Thompson 1986, Tercé-Laforgue et al. 1987, Domoney and Casey 1987, Dietrich et al. 1987, Datta et al. 1987, Dietrich et al. 1989, Goldberg et al. 1989, Harada et al. 1989). However, these types of analyses are just beginning for conifer seeds (eg. Pitel and Cheliak 1988b). Similar techniques are also being used to examine gene regulation associated with somatic embryogenesis (eg. Choi et al. 1987; Hahne et al. 1988).

One method to identify cDNA clones of developmentally regulated mRNAs is differential hybridization. By this method ^{32}P -labelled cDNA is made from mRNA prepared from the two stages of interest. Although most of the cDNA sequences will be similar in the two preparations, the cDNA synthesized from the RNA of the more differentiated (or treated with hormones, etc.) tissue will have additional sequences complementary to any new mRNA sequences. The two probes then screen replica filters of a cDNA library prepared from mRNA extracted from the induced-cell population. Any hybridization signal occurring specifically for the induced-cell cDNA probe should contain the cDNA of the induced mRNAs. However, this method is only suitable if a gene is expressed as a very abundant RNA in one cell type and is rare or absent in another. The limits of detection are mRNAs representing 0.1% of the total mRNA population. However, most genes are represented by only about 20 mRNA copies per cell (low abundance class) and thus a simple duplicate screening approach cannot be used. A typical eucaryotic cell has about 1 pg of mRNA (approximately 106 molecules) transcribed from about 15 000 different genes.

Palazzolo and Meyerowitz (1987) have described construction of the λ phage cDNA cloning vector λ SWAJ that allows directional cDNA cloning and selective amplification of either sense or antisense cRNA sequences. Such vectors have the SP6 and T7 promoters as well as several unique restriction sites. These vectors can be obtained from Promega Biotec under the names "Lambda GEM-2" and "Lambda GEM-4". The main purpose of developing these vectors was that subtractive hybridization (used in identifying rare stage or tissue-specific genes) requires as much as 20 μg of poly(A)⁺ RNA to drive the hybridization (Davis et al. 1984). However, with the above system 20 μg of sense cRNA is made from a cDNA library of one tissue while ^{32}P -labelled cDNA is synthesized from the mRNA prepared from the other tissue that has the unique mRNAs of interest. Only 1 μg of

mRNA is prepared from each tissue. After hybridization and subtraction the differential cDNA probe is used to screen an appropriate genomic or cDNA library. Other possible uses include: the identification of sequences present in one library and not another; the preparation of probes for *in situ* hybridization; and the construction of subtracted cDNA libraries in these vectors in order to generate high specific activity, single-stranded, antisense cRNA probes directly from DNA prepared from the whole subtracted library or from individual clones. The directional cDNA cloning scheme involves the use of an oligodeoxynucleotide primer-adaptor that contains the recognition site for the enzyme *Xba*I upstream from an oligo (dT) sequence. It is possible by this method to synthesize cDNA with an *Xba*I recognition sequence at the 3' end of each molecule. After second-strand synthesis, *Eco*RI linkers are added to each end. Double restriction digests with *Eco*RI+ *Xba*I results in ds cDNA with different sticky ends. The cDNA can then be directionally cloned into the vector which has the same two sticky ends. With this method RNAs representing only 0.01% of the total mRNA population can be identified (eg. Sargent 1987). Another procedure to obtain subtracted cDNA libraries is available from Invitrogen, and will be described briefly in Section 7.

2.2 Characterization of regulatory sequences of genes

Once a cDNA clone has been identified and sequenced it can be labelled (for example, by the use of random hexamers as described by Feinberg and Vogelstein (1983) or as purchased commercially in kit form from Amersham Inc. and others) and used to probe the homologous genomic library to obtain a corresponding genomic sequence. Thus, by comparing both sequences, the position of the introns, if any, and of the 5' and 3' flanking regions, can be determined. Some of these sequences may act as specific signals for controlling gene expression.

One of the promoter elements is the "TATA" box, which may play a role, together with specific regulatory proteins, in transcription by aligning RNA polymerase II. Some genes, such as that of the nuclear gene of tomato (*Lycopersicon esculentum*) encoding a chlorophyll a/b-binding protein of photosystem I (Cab-6B), have two TATA boxes (Pichersky et al. 1987). These two boxes are at nucleotides -97 to -94 and at -117 to -114, upstream from the ATG start codon. Two CCAAT boxes are found at nucleotides -145 to -141 and at -176 to -172, while a third is present 15-20 nucleotides below the downstream-most TATA sequence. It is unclear as to which of these sequences play a role in the transcriptional activity of the gene.

Many genes share these consensus sequences the position of which, relative to the transcription commencement site, are inflexible. Enhancers are another type of transcriptional regulatory element (see Grandbastien et al. 1986, Boronat et al. 1986). Unlike the promoter elements, the position and orientation of the enhancers relative to the transcription start site are flexible. They can greatly increase transcription of a linked gene from a position either upstream or downstream of a transcription unit (eg. in introns) in an orientation-independent manner (Rosenthal 1987). Also, enhancers do not share extensive sequence homology, as do the promoter elements. Some enhancers can be active in a wide variety of differentiated and undifferentiated cells, while the activity of others is tissue-specific. Thus, enhancers may regulate the expression of a gene in a tissue-specific manner (together with proteins that interact with the enhancers in a tissue-specific manner) and also regulate the amount of protein synthesized in a particular cell. In contrast, negative enhancers may be present that can repress transcription in a position- and orientation-independent manner.

The roles played by the various regulatory elements can be determined using reporter genes. The putative regulatory sequence is linked to a reporter gene whose transcription is detected after transfection into eukaryotic cells in culture. Some of these reporter genes can be bacterial genes coding for assayed enzyme activities such as CAT (chloramphenicol acetyltransferase) or β -galactosidase (eg. Rosenthal 1987). However, the GUS (or β -glucuronidase) assay system is gaining popularity (Jefferson 1987). The regulation of gene expression also depends on structural features of chromatin, such as nonhistone proteins and regions of Z-DNA that may possibly play a role in generating and maintaining regions of "open" chromatin, or DNaseI hypersensitive sites.

2.3 Studies with RFLPs (restriction fragment length polymorphisms)

From our jack pine and black spruce cDNA libraries we use radioactive random cDNA clones (each over 450 bp) for hybridization in Southern blotting experiments to genomic DNA of seven parental black spruce trees to detect RFLPs. In previous studies these variations in the size of restriction fragments homologous to a specific probe were ascribed either to point mutations in the DNA that would create or abolish restriction endonuclease sites, or to insertions/deletions that would alter their relative banding positions. According to Botstein et al. (1980) the number of RFLPs are essentially unlimited, they have no effect on phenotype, are codominant and

inherited in a Mendelian fashion, and there is little, if any, influence of the environment. One major application for nucleic acid probes is the development of physical and genetic maps. These maps, and the specific probes, have been either proposed for or are used for: (i) selection of characters (single and possible multi-gene) by exploiting linkages with phenotypic markers; (ii) varietal protection, assessment of levels of contamination, and more accurate descriptions of families and clones by genetic fingerprinting; and (iii) more accurate estimates of levels of genetic variation in natural and domesticated populations.

In a study with lettuce (*Lactuca sativa*) Landry et al. (1987a) observed that cDNA clones detected about 2.5 times more polymorphism than genomic DNA probes, and that the restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, and *Hind*III were the most efficient at detecting RFLPs. However, in some studies with humans, genomic clones detected polymorphism more frequently than did cDNA clones (Helentjaris and Gesteland 1983). Because genomic probes include both transcribed and non-transcribed sequences it may be expected that they would detect more variation than cDNA clones, because transcribed regions of DNA are more conserved than noncoding regions. Using random cDNA clones in studies with tomato, Bernatzky and Tanksley (1986a) indicated that the majority of structural genes are probably arranged in single loci and are present in low copy number. However, as indicated in another report (Bernatzky and Tanksley 1986b), genomic DNA probes may be needed to complete the construction of a linkage map if some regions of the genome are not detected by cDNA probes.

In plants, detailed genetic linkage maps using RFLPs are being constructed for species such as maize (*Zea mays*) (Helentjaris et al. 1986, Evola et al. 1986), tomato (Bernatzky and Tanksley 1986b), and lettuce (Landry et al. 1987b). The use of RFLPs as genetic markers are quite varied and include strain identification (eg. Evola et al. 1986), assessing genetic heterogeneity, evolutionary studies, the cloning of disease resistant genes (Landry et al. 1987b), marking genes controlling quantitative traits such as the soluble solids content of the tomato fruit (Osborn et al. 1987, Tanksley and Hewitt 1988), identification and mapping of duplicated DNA sequences with the maize genome (Helentjaris et al. 1988), and others (eg. Bernatzky and Tanksley 1986b, Landry and Michelmore 1987). In the longer term a genome mapping study may provide an opportunity to develop a genetics consulting application in tree breeding programs. However, this is an extremely large task and may take a full tree generation to complete before the map is of any real use. Once completed, the map

would constantly evolve as new traits are identified from selected pedigrees.

2.4 Gene expression regulated by hormones

With the recent advances in recombinant DNA methodology, studies can now be done to examine specific genes regulated by hormones as well as their protein products. Investigations have shown that auxin is involved in the expression of several genes in tissues associated with cell division, cell elongation and, possibly, cell differentiation (eg. Key 1987). In a study with mature hypocotyls of soybean (*Glycine max*) auxin was shown to increase synthesis of rRNA, RNA polymerase I activity, and ribosomal protein accumulation. This treatment results in the induction of cell division in the mature region. As auxin caused an eight-fold increase in translatable mRNAs for r(ribosomal)-proteins, Gantt and Key (1985) constructed a cDNA library to identify the r-protein mRNA clones. Analysis of Northern gels showed that probing with a cDNA encoding cytoplasmic ribosomal protein L13 resulted in a three- to eight-fold increase in the level of r-protein mRNA after 18 h (Key 1987). Auxin also regulated the level of specific mRNAs. In a study by Baulcombe and Key (1980), a cDNA library made from control poly(A)⁺ RNA was cloned and the colonies screened with labelled cDNAs made to poly(A)⁺ RNAs of control and auxin-treated tissue. cDNA clones for three different poly(A)⁺ RNAs that responded rapidly to auxin and were abundant in the control tissue (2.1% of total) were isolated. The concentration of each of these sequences decreased rapidly after 24 h of auxin treatment, most of this being prior to the start of auxin-induced cell division.

In cell elongation studies several auxin-regulated mRNAs and proteins have been identified. For example, with *in vitro* translation studies of poly(A)⁺ RNA and 2-D gels auxin was shown to selectively enhance the level of translatable mRNAs of excised soybean hypocotyl (Zurfluh and Guilfoyle 1982). Following auxin treatment of excised elongating hypocotyls, poly(A)⁺ RNA was isolated and cDNA libraries constructed (Walker and Key 1982). This experiment isolated two cDNA clones associated with cell elongation. The RNA sequences of these clones were rapidly depleted during incubation of excised sections in the absence of auxin. The addition of auxin to these tissues resulted in a rapid accumulation of both types of poly(A)⁺ RNAs. The RNA for one clone (JCW2) increased five- to ten-fold within 30-60 min. The authors indicated that the auxin-enhanced rate of cell elongation was the result of auxin-enhanced synthesis of specific "rate-limiting" mRNAs and their translation products. Genomic clones were isolated

that are homologous to some of the above clones and sequencing was initiated to examine the mechanism by which auxin regulates the expression of these genes and the function of the corresponding proteins (Key 1987). Antibodies can be made for cellular localization and other studies with the use of expression vectors.

Hagen et al. (1984) isolated four different cDNA clones to poly(A)⁺ RNAs that increase in soybean hypocotyl in response to auxin. These were selected from a cDNA library manufactured from poly(A)⁺ RNA of 2,4-D-treated seedlings. These changes induced by auxin are rapid and *in vitro* "run-off" transcription studies of nuclei isolated from control and auxin-treated tissues have shown that they are due to increased rates of transcription (Hagen and Guilfoyle 1985, Guilfoyle and Hagen 1987). After addition of auxin to excised plumules at least half maximal transcriptional rates for one clone (pGH1-4) occur within 15 min. while, for another clone (pGH3), a several-fold increase in transcription rate is observed within 5 min. of auxin application. The increased transcription rates on genes for two of the auxin-responsive mRNAs are increased by a variety of auxins such as IAA, NAA, and 2,4,5-T and also by other chemicals such as CdCl₂. However, ethylene (the synthesis of which may be stimulated by applied auxins) does not induce the "auxin response". As the induction of transcription is rapid, selective (few cDNA clones involved) and direct (independent of protein synthesis) transcription events may occur close to the primary site of action of auxin (Hagen 1987).

2.5 Other studies

The literature on the use of cDNA techniques is extensive and we provide the reader with only a representative selection for further reference.

Hiesel and Brennicke (1987) first constructed a cDNA library to obtain information about actively transcribed genes before characterizing plant mitochondrial DNA, in which the genes are often separated by large non-coding regions. cDNAs from all known genes were present.

When investigating herbicide tolerance in maize, Wiegand et al. (1986) isolated cDNA clones for the major polypeptide subunit of glutathione-S-transferases (GSTs), termed GST A. In maize these enzymes conjugate glutathione to several major classes of pre-emergent, selective herbicides. Among other studies, the authors used this clone as a probe for Northern analysis to examine mRNA levels following treatment with safeners.

Fruit ripening, which sees changes in colour, texture, and aroma, is thought to involve alterations in specific gene expression and, in turn, alterations in enzyme activity. cDNA libraries have been used to identify and characterize ripening-specific cDNA clones (eg. Morgens et al. 1987, Piechulla and Gruissen 1987, Della Penna et al. 1987, Maunders et al. 1987). For example, as reported by Della Penna et al. (1987), a 2000-fold increase in levels of polygalacturonase mRNA was observed during ripening following the use of cDNA clones for the enzyme. Although agents responsible for the regulation of certain genes during fruit development and ripening (eg. hormones, DNA sequence, binding proteins, environment) are still being studied, we know that the expression of genes coding for the small subunit of ribulose-1,5-bisphosphate carboxylase (rbcS) and the light harvesting chlorophyll a/b binding protein (cab) are stimulated by light. In the review article by Kuhlemeier et al. (1987) various results associated with both the cis-acting and trans-acting elements involved in the transcription of light-regulated genes are described. During fruit development and ripening, regulatory mechanisms are present for both nuclear and plastid genes (eg. the Q_B binding protein, psbA) and these are highly coordinated at this time. Using cDNA cloning techniques, Titlgen et al. (1986) isolated numerous genes for the nuclear-encoded complement of thylakoid membrane polypeptides from spinach.

cDNA cloning has been useful for examining the induction of gene expression following various types of environmental stress. For example, in the review article by Sachs and Ho (1986), anaerobic-specific cDNA clones for maize were identified. Among other uses, these were used as probes to measure gene expression during anaerobic treatment. The kinetics of mRNA increase were shown to be the same for Adh1 and Adh2; the level for both appeared after 90 min. of anaerobic treatment and continued to increase until levelling off at 50-fold above the aerobic level after 5 h of anaerobiosis. For Adh1 the level declined after 49 h and for Adh2 it declined after 10 h. With further characterization of DNA sequences associated with the encoding regions and regulation of expression of stress proteins, there is a possibility that genes coding for stress proteins from tolerant plants could be introduced to sensitive plants to withstand stress conditions.

Investigations of plant gene expression in response to pathogens or elicitors involve the use of molecular biology. For example, genes for certain pathogenesis-related (PR) proteins (host-coded proteins induced by viruses, bacteria, and fungi as well as by abiotic elicitors such as uv light and some

chemicals) have been characterized using cDNA libraries (eg. Collinge and Slusarenko 1987). Sequence analyses for several PR cDNA clones have been examined by Matsuoka et al. (1987). One purpose of identifying resistance-specific cDNAs is to probe genomic libraries of host DNA in order to understand the nature of upstream regulatory sequences controlling gene expression. Experiments with other types of responses to pathogens or elicitors, such as the level of phenylpropanoid metabolism or increased endochitinase activity, have also used cDNA cloning techniques. Further studies in this area could involve the use of transgenic plants containing cloned defence-related genes, as well as the use of gel retardation assays, DNase hypersensitivity mapping, and DNA "footprinting" techniques to study the nature of the relevant regulatory sequences and the trans-acting regulatory elements (Collinge and Slusarenko 1987).

In studies by Peña-Cotes et al. (1988) of potato (*Solanum tuberosum*), both cDNA and genomic clones encoding proteinase inhibitor II, a trypsin/chymotrypsin inhibitor, have been isolated. These clones were used to follow the induction of expression of the proteinase inhibitor II genes at the RNA level following wounding.

Many different studies involving the use of cDNA techniques were presented at a recent meeting, with abstracts published in the Journal of Cellular Biochemistry (UCLA Symposia on molecular and cellular biology, J. Cell. Biochem. Supplement 12C, 1988, Alan R. Liss, Inc., New York). Among these studies were an analysis of gene expression with flower development; the induction of genes in water stressed cereal seedlings by ABA; the identification and characterization of tissue-specific sequences; gene activation in fruit tissues induced by ethylene; characterization of pollen-specific cDNA clones; identification of cDNA clones from *Larix laricina* for the small subunit of ribulose biphosphate carboxylase/oxygenase (rbcS) and for the light harvesting complex (chlorophyll a/b-binding) protein (LHCP); gene expression of cellulase activity with fruit ripening; analysis of genes induced by heat shock; characterization of regulation of enzyme activity; identification of haemoglobin-related sequences in non-nodulating species; and, analysis of gene expression with meristem formation.

3 Extraction of total plant RNA

3.1 Minimizing ribonuclease activity

Ribonucleases are generally present in cells and tissues and are very stable. Improper handling of ribonucleases can contaminate glassware, other implements, and solutions. Glassware intended for RNA studies should, therefore, be new and used only for this purpose. After the usual detergent wash glassware should be put in a 0.1% solution of diethyl pyrocarbonate (DEPC, a nonspecific inhibitor of RNase), autoclaved to destroy the remaining DEPC, and then baked in an oven at 250°C for at least 3 h. Use sterile disposable plasticware. Where possible we use sterile, individually-wrapped "transfer pipettes", pipette tips, etc. We also use sterilized 15 mL and 50 mL polypropylene screw cap conical tubes (eg. Sarstedt), say, for phenol extractions of the RNA mixtures. Eppendorf tubes, pipette tips, and other non-sterile plasticware must be autoclaved before use. Plastic or latex disposable gloves are recommended at all times.

Solutions are first treated with DEPC, and the DEPC is then removed prior to use as it reacts with adenine residues of RNA, which will inactivate *in vitro* translation reactions. The solutions are adjusted to 0.1% in DEPC, shaken thoroughly or stirred vigorously for 10 min. and then autoclaved. The decomposition products, CO₂ and ethanol, are volatile and thus easily removed from the hot solutions. As DEPC reacts with Tris, these buffers should be prepared with DEPC-treated autoclaved water, and then reautoclaved. If possible, dry chemicals should be purchased and reserved only for RNA work, and weighed out with baked spatulas.

As some cells or tissues may contain high levels of endogenous ribonucleases, various specific and non-specific inhibitors of ribonuclease can be included during RNA isolation, during cDNA preparation, or other reactions involving mRNA. For example, RNasin (placental ribonuclease inhibitor) is a potent inhibitor of RNase that is useful for *in vitro* studies such as nuclear run-on transcription, translation in cell-free systems, and reverse transcription of mRNA. Its activity requires the addition of at least 1mM dithiothreitol, and it can be easily removed with phenol. The preparation of another specific inhibitor, vanadyl-ribonucleoside complexes, is described by Maniatis et al. (1982). The use of nonspecific inhibitors such as polyvinyl sulfate, heparin, and 2% solutions of clays (bentonite or macaloid) is not completely satisfactory (Blumberg 1987). Either pronase or proteinase

K (especially with the addition of denaturing agents such as 1-2% SDS) is useful to digest ribonuclease and other proteins. Intact RNA can be isolated from tissues rich in RNase in the presence of chaotropic agents such as guanidine thiocyanate, together with reducing agents such as β -mercaptoethanol.

3.2 RNA extraction using phenol

Phenol should be redistilled if it is crystalline or has a yellow or pink colouration. These coloured products (eg. quinones, diacids) can cause cleavage of phosphodiester bonds and cross-linking of DNA strands. As the rate of phenol oxidation increases with pH (eg. when equilibrated with buffers greater than pH 8) these reagents should be used fresh and not stored. The addition of the antioxidant 8-hydroxy-quinoline (0.1%, w/v) to the phenol is helpful as it retards the rate of phenol oxidation, the yellow colour results in easier location of the phases, and it is a partial inhibitor of ribonuclease. In some cases the phenol is equilibrated with TE buffer (3 mL TE per 10 mL phenol) and kept in the dark at 4°C for up to one month. For RNA studies, the phenol is usually equilibrated with Tris-HCl buffers (see Appendix I).

The addition of chloroform, that is, extractions with phenol (TE-saturated)-chloroform-isoamyl alcohol (24:24:1, v/v/v), results in improved efficiency of RNA (and DNA) extractions. Chloroform assists in the dissociation of nucleic acid from protein, helps remove lipids, and its high density enhances separation of the phases.

The pH of the extraction buffer should be checked carefully because RNA is easily lost. If phenol alone is used to extract RNA at a pH below 7.6, poly(A)⁺ mRNA is lost in the organic phase and some poly(A)⁺ tracts may also be cleaved. To ensure the poly(A)⁺ RNA separates into the aqueous phase, extraction should be carried out at pH 9, or between pH 5-9 in combination with chloroform or SDS. At pH 8 or higher both DNA and RNA will be in the aqueous phase, while at pH 5-6 DNA is selectively retained in the organic phase and interphase.

Although extractions of mRNA with high salt concentrations (0.2-0.5M NaCl) can reduce ribonuclease action, optimal RNA yields occur at concentrations of less than 0.2M. The maximum solubility of RNA occurs at 0.15M Na⁺, and at this point non-specific RNA aggregation is reduced. Low NaCl concentrations should only be used if endogenous ribonuclease is not a problem or if suitable ribonuclease inhibitors are present (Wallace 1987).

The ionic detergent SDS dissociates proteins from nucleic acids and mildly inhibits nucleases. Effective release of RNA occurs with brief exposure of the extract to high concentrations of SDS (0.1-2.0%) at 10°C or higher. The presence of EDTA prevents aggregation of nucleic acids to each other and to proteins, which happens when Mg^{2+} is present. To further dissociate proteins and reduce the amount of protein interference, the extract can be incubated with proteinase K (50-200 µg/mL, final concentration) at 37°C for 30 min. in the presence of SDS. The addition of powerful ribonuclease inhibitors such as vanadyl-ribonucleoside complexes and RNasin to RNA extracts prior to phenol extraction is sometimes required to extract intact mRNA from tissues with high ribonuclease levels (Wallace 1987). Although RNA should be extracted as quickly as possible at 0-4°C with cold buffers to minimize nuclease activity, the addition of SDS requires extraction at 10°C or higher. If endogenous ribonucleases are present in low concentrations, phenol extractions can be performed at 50-60°C.

3.3 Extracting with guanidinium thiocyanate

Guanidinium thiocyanate and guanidinium isothiocyanate (the guanidinium salts of thiocyanic acid) are actually the same compound in solution. At 4M it is a strong protein denaturant that is very effective in inhibiting RNase when isolating RNA from tissues rich in RNase. The compound is harmful if inhaled or swallowed and should be used in a well-ventilated area.

A critical stage in the extraction is removal of the homogenate and washing the RNA cushion surface after ultracentrifugation. Remember that although the RNases are denatured in the thiocyanate, once this is diluted out, they can renature and regain activity. One method is to cut the top portion off the centrifuge tube. The surface of the cushion should be washed thrice with sterile dH_2O . The pellet should have a clear, lens-like appearance.

Denaturation with guanidinium thiocyanate is enhanced by including a reductant such as 2-mercaptoethanol or dithiothreitol to break intramolecular protein disulfide bonds. Some reports suggest that DEPC not be used due to its reactivity towards nucleic acids. Frozen tissues should be pulverized in liquid N_2 , then homogenized (with a Polytron or Waring blender) immediately without thawing after adding the guanidinium solutions. For some tissues the use of a motorized pestle resulted in much degraded RNA. One method of separating RNA from the other macromolecules is through selective precipitation by

ethanol or lithium chloride. Problems may occur with this procedure if the RNA concentration in the homogenate is low; if so, it may not precipitate. For good recovery of RNA, it should be centrifuged through a dense solution of cesium chloride. The RNA pellet may have to be further purified with phenol extraction. Proteinase K digestion is useful if peptides are covalently linked to the RNA. The use of the Polytron homogenization procedure shears the nuclear DNA, preventing the formation of an impenetrable DNA mat which blocks sedimentation of the RNA.

3.4 Extraction of total RNA

RNA extraction procedures may vary with the species and/or tissue being used. We describe below several methods that were suitable for our extractions with conifer seed material. Although we have used the following five extraction protocols successfully for seed material, method no. 1 was most frequently employed. During extraction of mRNA it is important to minimize ribonuclease activity during the initial stages and to avoid the accidental introduction of trace amounts of ribonuclease from glassware and solutions. Sterile, disposable plasticware is essentially free of RNase and can be used without pretreatment. However, glassware should be treated by baking at 250°C for 3 or more hours or at 180°C for at least 8 h. In addition, glassware can be treated for 12 hours at 37°C with a solution of 0.1% diethylpyrocarbonate. This is a strong but not absolute inhibitor of RNase. Traces of diethylpyrocarbonate should then be removed by heating to 100°C for 15 minutes.

We remind you to wear gloves at all stages during the preparation of materials and solutions, and during all manipulations involving RNA. All solutions should be prepared using baked glassware, glass-distilled autoclaved water, and dry chemicals handled with baked spatulas. Wherever possible, solutions should be treated with 0.1% DEPC for at least 12 hours and autoclaved for 15 minutes. However, diethylpyrocarbonate cannot be used to treat solutions containing Tris. All diethylpyrocarbonate must be destroyed because it modifies RNA. It is also a potent alkylating agent and must be used with caution.

Extraction method No. 1

The following method is essentially as described by Kreibel (personal communication) and Whitmore and Kreibel (1987):

1. Grind 10 g of tissue with liquid N₂ and transfer to a suitable container.

2. Add the following extraction buffer (25 mL) after heating buffer in a boiling water bath to about 65°C, then grind in a Polytron or Waring blender for 1 min. at the highest speed:

0.2M sodium borate; 30mM EGTA; 1% SDS; 5 mM dithiothreitol

To 80 mL of water add:

7.6 g sodium borate

1.14 g EGTA

1.0 g SDS

0.08 g dithiothreitol

Adjust pH to 9.0 with 1N NaOH and dilute to 100 mL. Add 50 µL of DEPC and store at 4°C.

For mature tissues of larch 0.1% Triton X-100 was substituted for SDS in all stages (Hutchison et al. 1988).

3. Filter the mixture through one layer of Miracloth into a 50 mL polypropylene centrifuge tube. Add self-digested Proteinase K (13 mg in 2 mL of 10mM Tris-acetate, pH 7.5, buffer for 30 min.) and incubate 1 h at 37°C.
4. Add 2 mL of 2M KCl to precipitate SDS. Mix well and centrifuge at 10 000 rpm for 10 min. at 4°C.
5. Put supernatant in another tube, add 1/4 vol. of 10M LiCl, and mix well. Freeze 20 min. at -70°C and then place at 4°C overnight.
6. Centrifuge at 10 000 rpm for 20 min. and then wash the pellet twice with 2M LiCl.
7. Suspend the pellet in a few mL of 2M LiCl and transfer to a Dounce tissue grinder. Break up the clumps until homogeneous, and transfer to a centrifuge tube. Spin at 10 000 rpm for 10 min.
8. Repeat Step 7 two or three times.
9. Wash pellet with 2M LiCl, drain tube, and centrifuge briefly to collect any remaining LiCl at the bottom of the tube. Remove this with a sterile pipette tip.

10. Dissolve pellet in 2 mL sterile H₂O and centrifuge for 10 min. at 10 000 rpm. Transfer the clear solution to a clean 15 mL tube.

11. Add 50 µL/mL of 3M sodium acetate and 2.5 vol. cold absolute ethanol. Place at -20°C for at least 2 h.

12. Centrifuge at 10 000 rpm for 10 min. and add 2 mL sterile phenol extraction buffer (10mM Tris-HCl, pH 9.0; 1mM EDTA; 0.01% SDS) to the pellet.

13. Add an equal amount of buffer-saturated phenol. Shake several min. and centrifuge at 4 000 rpm for 10 min. Remove top phase and save.

14. To the bottom (phenol) phase add an equal vol. of phenol extraction buffer, pH 9.0; shake and centrifuge as above. Combine the two aqueous phases.

15. Extract the combined phases with an equal vol. of phenol/chloroform/isoamyl alcohol (24:24:1). Shake for several min. and centrifuge as above.

16. Re-extract with phenol/chloroform two or three times more.

17. Add 50 µL/mL of 3M sodium acetate and 2.5 vol. cold absolute ethanol. Put at -70°C for 15 min. and transfer to -20°C overnight.

18. Centrifuge at 10 000 rpm for 10 min. and discard the supernatant. Add 2 mL of 10mM Tris-HCl (pH 7.6) buffer, containing 1mM EDTA, and 0.1% SDS. Transfer solution to a 15 mL centrifuge tube and add 50 µL/mL of 3M sodium acetate and 2.5 vol. cold ethanol. Put at -20°C for at least 2 h. To remove any impurities, such as carbohydrates, use Sigmacell Type 50 Cellulose Column as detailed below.

Sigmacell Type 50 Cellulose Column

1. Put a few grams of Sigmacell Type 50 cellulose into a 25 mL flask. Wash with 1X binding buffer. After a few minutes, decant. Repeat the wash a few times. Pipette the slurry into an 11 mL disposable Poly-Prep™ chromatography column (Bio-Rad) until the packing is 4 cm in length. Run a few mL of 1X binding buffer through the

column and adjust flow rate to 1 drop every 10-12 seconds.

2. Centrifuge the sample at 10 000 rpm for 10 minutes and discard supernatant. Add 1 mL elution buffer, pH 7.6 and heat to 60°C for 5 min. Cool in an ice bath. Add 1 mL 2X binding buffer. Load sample onto the column and begin collection as soon as 1.5 mL has been discarded. Collect 4 mL with 1X binding buffer in a 15 mL sterile plastic tube.
3. Transfer sample to a 50 mL centrifuge tube. Add 50 µL/mL of 3M sodium acetate and 2.5 volumes of cold 95% ethanol. Place at -20°C for at least 2 hours.

Extraction method No. 2

This method is as described by Martin and Northcote (1981).

1. The tissue is ground with liquid N₂ and allowed to thaw in the following extraction buffer (3 mL/g tissue)

50mM Tris-HCl, pH 9.0
150mM NaCl
5mM EDTA
5% (w/v) SDS

2. Homogenize for 1 min. in a Waring blender, or grind to a fine slurry with mortar and pestle using acid washed sand.
3. Filter through Miracloth and add an equal volume of redistilled, buffer-saturated phenol/chloroform (1:1). Stir at 4°C for 10 min.
4. Centrifuge at 4 000 rpm for 5 min. and save the aqueous phase. Re-extract the organic phase with 0.25 vol. washing buffer (20mM Tris-HCl, pH 9.0; 2mM EDTA) and centrifuge as above.
5. Combine the two aqueous phases and stir with an equal vol. of phenol/chloroform at 4°C for 5 min. Centrifuge to separate the phases.
6. Re-extract with phenol/chloroform at least twice again.
7. The final aqueous phase is made to 0.2M with solid NaCl and RNA is precipitated with 2.5 vol. ethanol at -20°C overnight.

8. Centrifuge at 12 000 rpm for 10 min. at 4°C and wash at least three times with 3M sodium acetate (pH 5.5) to remove contaminating polysaccharides and low molecular weight RNA (4S and 5S).
9. Wash pellet with 70% ethanol and dry by vacuum. Dissolve in sterile dH₂O and reprecipitate RNA with two vol. of 3M LiCl at 4°C for 16 h.
10. Collect the precipitate, wash with 70% ethanol, and vacuum-dry.
11. Dissolve pellet in 0.3M NaCl, and remove any insoluble material by centrifugation.
12. Add 2.5 vol. ethanol to the supernatant. After leaving overnight at -20°C, the final RNA pellet should be dissolved in sterile dH₂O to a conc. of 10 mg/mL and stored at -70°C.

Extraction method No. 3

This method was developed by Logemann et al. (1987) mainly for extracting RNA from wounded potato tubers which contained high levels of polysaccharides and nucleases. However, other tissues can also be used. We have obtained good results with seed and callus tissues of conifers. This method was also used to extract RNA from small amounts of tissue (0.2-1 g) by homogenizing directly in the Eppendorf tube with a stainless steel rotating pin that fits exactly at the bottom of the tube. With this "miniprep" procedure large numbers of samples (about 10-20/day) can be prepared. For tuber and leaf tissues of potato Logemann et al. obtained yields of up to 171 and 436 µg RNA/g tissue, respectively. Their RNA preparations were intact and of sufficient purity and quality for *in vitro* translation, cDNA cloning, and other studies.

1. Grind tissue to a fine powder with liquid N₂.
2. Homogenize (eg. with a Polytron blender) following addition of two vol. of the following extraction buffer

6M guanidine thiocyanate
20mM Mes (4-morpholineethansulfonic acid)
20mM EDTA
50mM mercaptoethanol
Final pH of 7.0

3. Centrifuge at 10 000 rpm for 10 min.
4. Pass supernatant through one layer of Miracloth.
5. Extract three times with one volume of phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v). The phenol was equilibrated twice with 2M Tris-HCl, pH 8.0, and stored with 0.1 mM Tris-HCl, pH 8.0.
6. Centrifuge for 45 min. at 10 000 rpm at room temperature to separate phases.
7. The aqueous phase is mixed with a precooled 0.7 vol. of ethanol and 0.2 vol. of 1M acetic acid for precipitating the RNA, leaving DNA and residual proteins in the supernatant. Keep at -70°C for 1 hour or at -20°C overnight.
8. Centrifuge at 10 000 rpm for 10 min. and wash at least twice with sterile 3M sodium acetate (pH 5.2) at room temperature. Centrifuge for 5 min. at 10 000 rpm. RNA will stay as a pellet, while contaminating polysaccharides and low molecular weight RNA will be in solution.
9. Wash RNA pellet with 70% ethanol. Store the RNA in ethanol at -70°C.

Extraction method No. 4

This method is essentially as described by Lay-Yee et al. (1987) for extraction of cotyledons of *Pharbitis nil* Choisy cv. Violet. The RNA was suitable for *in vitro* translation studies.

1. 5 g of tissue was ground to a powder and then added to 35 mL of the following buffer

6M guanidine thiocyanate
 10mM EDTA
 200mM Tris-HCl, pH 8.0
 8% (v/v) 2-mercaptoethanol
2. Homogenize in a blender at room temperature.
3. Centrifuge at 10 000 rpm for 20 min., and then add seven volumes 4M LiCl to the supernatant. Keep overnight at 4°C.
4. Collect the precipitated RNA by centrifugation at 11 000 rpm for 90 min. Resuspend and centrifuge pellet with 3M LiCl at least once.

5. Resuspend pellet in the following solubilization buffer:

10mM Tris-HCl (pH 8)
 5 mM EDTA
 1% SDS

6. Extract three or more times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v).
7. To the aqueous fraction add 1/10 vol. 3M sodium acetate and 2.5 volumes cold ethanol. Store at -80°C.
8. Before isolation of poly(A)⁺ RNA, the total RNA preparation is dissolved in the above solubilization buffer, brought to 0.5M with NaCl, heated to 65°C for 5 min., cooled rapidly in an ice bath to room temperature and then passed over a cellulose CF11 (Whatman) column equilibrated with 20mM Tris-HCl (pH 7.6) containing 0.5M NaCl, 1mM EDTA, and 0.1% SDS, in order to remove polysaccharide contaminants.

Extraction method No. 5

This method was described by Reeves et al. (1986) for extracting total RNA from wheat (*Triticum aestivum* L.) seeds.

1. For each g of seed, grind in liquid N₂ and suspend in 10 mL of buffer

4M guanidine HCl
 10mM Tris-HCl, pH 8.0
 10mM EDTA
 0.5% (w/v) Sarkosyl
 3% (w/v) PVPP
2. Extract with phenol/chloroform.
3. Precipitate nucleic acid at -20°C for 2 h by addition of 1/100 vol. 3M sodium acetate, pH 4.8 and ethanol.
4. Centrifuge at 12 000 rpm for 10 min. and suspend pellet in 1% Sarkosyl containing 100 µg/mL proteinase K using a glass-Teflon homogenizer and warming to 50°C.
5. For each 2.5 mL, 1 g CsCl is added and this solution is centrifuged at 200 000 rpm for 16 h at 20°C.

6. Dissolve pellet in 10mM Tris-HCl (pH 7.0), 100mM NaCl, 0.5% SDS, and precipitate with ethanol.

Extraction method No. 6

The method described by Hughes and Galau (1988) was developed mainly to extract RNA from leaves and pollen of cotton (*Gossypium hirsutum*). However, the method is suitable for other tissues and species unsuitable for phenol or guanidinium extraction. The authors indicated that for cotton tissues there was irreversible binding of RNA to the homogenized leaf tissue when phenol or guanidinium was present. In their modified method large amounts of RNA are present in isopropanol precipitates of the crude extracts. RNA yields are increased with use of deoxycholate, NP-40, and high concentrations of LiCl. The use of acidic potassium acetate improves RNA recovery, removes visible impurities, and decolourizes the clarified homogenate.

1. Homogenize (eg. with a 30-mL Duall tissue grinder; Kontes, Vineland, NJ) 0.8 g fresh weight of leaf tissue with 10 mL ice cold homogenization buffer:

200mM Tris-HCl, pH 8.5 (at room temperature)
1.5% lithium dodecylsulfate
300mM LiCl
10mM Na₂ EDTA
1% w/v sodium deoxycholate
1% v/v NP-40
5mM thiourea
1mM aurointricarboxylic acid
10mM dithiothreitol

Solutions are prepared from stocks filtered through 0.45 μ m nitrocellulose filters and treated by autoclaving after shaking 30 min with 0.1% DEPC.

Transfer above mixture to a 15 mL plastic conical centrifuge tube and allow to freeze slowly at -80°C.

2. Place in a 37°C bath until just thawed. Immediately put on wet ice and add 1/3 volume 8.5M potassium acetate, pH 6.5. Mix by inversion and keep on ice for 15 min. Centrifuge at 5000 rpm for 20 min. Transfer the supernatant through one layer of Miracloth into a new 15 mL conical centrifuge tube. Add 1/9 volume 3.3M sodium acetate, pH 6.1, and half final volume cold isopropanol. Mix and store at -20°C for at least 1 h.

3. Centrifuge at 5000 rpm for 30 min. and resuspend pellet in 800 μ L TE and transfer to a 1.5 mL microcentrifuge tube. Add 1/4 volume of 10M LiCl to 2M and incubate on ice for 5-12 h.
4. Centrifuge at 10 000 rpm for 30 min. and resuspend pellet in 200 μ L TE. Add 1.5 volumes 5M potassium acetate (pH not adjusted) mix, and put on ice for 3 to 5 h.
5. Centrifuge at 10 000 rpm for 30 min. and resuspend the pellet in 100 μ L TE and incubate on ice for 1 h, vortexing occasionally. Centrifuge as before and remove supernatant to a new microfuge tube. Add 1/9 volume 3.3M sodium acetate, pH 6.1 and two volumes ethanol. Mix and place at -20°C for at least 1 h. If desired the above supernatant can be extracted with phenol/chloroform and chloroform.
6. Centrifuge at 10 000 rpm for 30 min. and drain supernatant. Vacuum dry the pellet and dissolve in 50 to 100 μ L TE.

3.5 Other RNA extraction procedures

There are a variety of ways other than the above to extract RNA from plant tissues using different types of buffer, different homogenization methods, and changing the sequences of procedures. For example:

To extract RNA from alfalfa (*Medicago falcata*) seedlings, Mohapatra et al. (1987) used a low pH extraction buffer (200mM sodium acetate, pH 5.2, 10mM EDTA, 1mM SDS). After grinding seedlings in liquid N₂, the powder was extracted with two volumes of the buffer, together with two volumes of phenol (both at 60°C). After centrifugation at 6000 rpm, the aqueous phase was further extracted with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol. The RNA was precipitated overnight at 4°C with LiCl (final conc. of 2M) followed by a further wash with 2M LiCl and two washes of 70% ethanol.

Total RNA was extracted from rice (*Oryza sativa*) embryos by grinding in a chilled mortar with a buffer containing 0.02M Tris-HCl (pH 7.6), 0.5M KCl, 2mM MgCl₂, 0.5% SDS, 0.5% bentonite, and 0.05M β -mercaptoethanol (Ghosh and Chaudhuri 1984). After phenol-chloroform extractions and ethanol precipitation of the aqueous phase, the RNA pellet was dissolved in 0.01M Tris-HCl (pH 7.6) containing 0.3M NaCl and treated with DNase P (50 μ g/mL) at 37°C

for 1 h. Following further phenol/chloroform extractions, the RNA in the aqueous phase was precipitated with ethanol and dissolved in buffer prior to oligo (dT) cellulose chromatography.

In a study by Chrispeels et al. (1984) with *Phaseolus vulgaris*, 1-3 g of cotyledons were ground with liquid N₂, transferred to another mortar at room temperature and ground to a slurry in 10 mL of 0.33M sucrose, 30mM MgCl₂, 60mM KCl, 0.1M Tris-HCl (pH 8.5), 2mM EDTA, and 1% iodoacetic acid. After centrifugation at 13 000 rpm for 10 min., SDS was added to 0.5%, and the supernatant extracted once with an equal volume of phenol, twice with phenol/chloroform, and once with chloroform. To remove polysaccharides, the aqueous phase was made to 0.3M sodium acetate, 10% ethanol, incubated on ice for 1 h, and then centrifuged at 20 000 rpm for 20 min. The aqueous phase was carefully removed and the RNA precipitated with 2.5 volumes of ethanol. After centrifugation the pellet was resuspended in H₂O and the RNA reprecipitated with four volumes of 3M sodium acetate, pH 5.5. The final precipitate was washed with 3M sodium acetate and 70% ethanol.

For extraction of total RNA from elongating soybean (*Glycine max*) hypocotyls, Walker et al. (1985) homogenized the tissue with a Polytron in an extraction buffer containing 10mM Tris-HCl (pH 8.8), 50mM NaCl, 6% (w/v) p-aminosalicylic acid, 2% (w/v) triisopropyl-naphthalenesulfonic acid, and 6% (v/v) butanol. This was followed by repeated phenol/chloroform extractions. This protocol was similar to that described by Parish and Kirby (1966).

For extraction of total RNA from cotyledons of mustard (*Sinapis alba*) seedlings, Sharma and Schopfer (1987) added the liquid N₂-ground powder to NETS buffer (0.12 M NaCl, 10 mM EDTA, 25 mM Tris-HCl (pH 7.4), 15 g/L SDS, 0.2 g/L proteinase K) at a ratio of 10 mL of buffer per g fresh tissue. The homogenate was incubated at 37°C for 1 h with occasional stirring, and then extracted a few times with phenol/chloroform. After ethanol precipitation of the RNA, residual phenol was removed from the sample by dissolving the pellet in ETS buffer (0.5 mM EDTA, 20 mM Tris-HCl (pH 7.4), 2 g/L SDS) and reprecipitation with ethanol. DNA was removed by repeated extraction with 3 mM sodium acetate, pH 5.2.

To extract RNA from pine (*Pinus thunbergii*) embryos Yamamoto (1982) homogenized the embryos in a Waring blender for 3 min. with 50mM Tris (pH 9.0), 1% SDS, and an equal volume of water-saturated phenol. After stirring for 5 min. and centrifugation at 900 rpm for 5 min. the aqueous phase was extracted

three times with phenol. The phenolic and middle phases were re-extracted as well. After precipitating the RNA with ethanol and then dissolving the pellet in water, DNA and low molecular weight RNA were removed after precipitation of the high molecular weight RNA with 0.2 volumes of 10M LiCl overnight at 0°C.

Radish (*Raphanus sativus*) seed tissue was ground to a powder with liquid nitrogen before adding the extraction buffer: 0.1M glycine-NaOH (pH 9.0), 0.15M NaCl, 1% desoxycholate, 0.1% SDS, and proteinase K to 60 µg/mL (Aspart et al. 1984). An equal volume of phenol/chloroform was added, the mixture homogenized for 2 min. in a Waring blender, and then stirred 15 min. at 4°C. After centrifugation for 15 min. at 8 000 rpm, proteinase K was added to the supernatant (20 µg/mL) and the mixture stirred with phenol/chloroform for 15 min. at 45°C. After centrifugation, further phenol/chloroform extractions, and ethanol precipitation the pellet was dissolved in sterile 20mM Tris-HCl (pH 9.0), 0.15M NaCl, and 0.1% SDS. To this 4M NaCl (pH 5.5) or 4M sodium acetate (pH 7.0) was added to a final concentration of 3M to precipitate the high molecular weight RNA after 2-5 h at 0°C. This step removed unidentified translation inhibitors as well as DNA, low molecular weight RNA, and polysaccharides.

Tissues of *Arabidopsis*, ground with liquid nitrogen, were added to 10 vol. of 10mM Tris-HCl (pH 8.3), 150mM NaCl, 1mM EDTA, and 0.1% SDS (Dolferus et al. 1985). An equal vol. of phenol/chloroform was added and extracted with a Waring blender. After centrifugation for 30 min. at 10 000 rpm at 10°C, the aqueous phase was re-extracted several times with phenol/chloroform. After precipitation with ethanol, the RNA pellet was dissolved in 10mM Tris-HCl (pH 7.5), 1mM EDTA, and 0.5% SDS. High molecular weight RNA was then precipitated by addition of an equal volume of 4M LiCl and keeping overnight at -20°C.

Leaf tissue of tomato (*Lycopersicon esculentum*) was ground to a powder with liquid nitrogen and then added to four vol. of 5% (w/v) 4-aminosalicylic acid, 1% (w/v) triisopropyl-naphthalene sulphonic acid, 5% (v/v) phenol mixture (400 mL H₂O, 140 mL m-cresol, 1 g 8-hydroxyquinoline added to 1 kg phenol), and 50mM Tris-HCl, pH 8.5 (Smith et al. 1985). The homogenate was extracted with an equal vol. of phenol/chloroform, shaken at room temperature, and centrifuged. This was followed by further phenol/chloroform extractions and a final precipitation of the high molecular weight RNA with 3M sodium acetate, pH 6.0.

De-embryonated seeds of barley (*Hordeum vulgare*) were frozen in liquid nitrogen and ground to a powder in a precooled Waring blender (Mozer 1980). This was added to a buffer of 100mM sodium glycinate, pH 9.5, 100mM EDTA, 100mM NaCl, 1% SDS, 1% PVPP, 5mM 2',3'-AMP, 10mM mercaptoethanol, and 10 µg/mL proteinase K. After centrifugation the supernatant was extracted twice with phenol/chloroform. To this were added, 1/10 vol. of 1M sodium acetate, pH 5.0, and enough acetic acid to lower the pH to 5.0. After centrifugation the pellet, containing RNA and polysaccharides, was suspended in 20mM Tris-HCl, pH 7.6, and 0.5% SDS. This solution was heated to 60°C for 5 min., rapidly cooled, and an equal vol. of 1M sodium acetate added. The sample was then passed through a Sigmacell type 50 cellulose column to remove the large quantity of carbohydrate bound nonspecifically to cellulose in high salt and eluted off in low salt.

For extraction of RNA from different tissues of tomato (fruit, stem, root, leaf, seedling), 50 g of frozen and ground material was added to 50 mL buffer: 0.35M sorbitol, 50mM Tris-HCl, pH 8.0, 25mM EDTA, 15mM 2-mercaptoethanol, 2mM dithiothreitol, 0.1% PVP, and 5mM aurointricarboxylic acid (Piechulla et al. 1986). One-tenth vol. of chloroplast lysis buffer (5% sodium laurylsarcosinate, 50mM Tris-HCl, pH 8.0, 25mM EDTA) was added and the mixture kept for 15 min. at room temperature. This was followed with three phenol/chloroform extractions, and precipitation of RNA with 1/10 vol. of 5M ammonium acetate (pH 5.2) and two vol. of ethanol. The pellet was suspended in TE and 10M LiCl added to a final concentration of 2M to precipitate high molecular weight RNA.

Frozen leaf tissue of *Mesembryanthemum crystallinum* was ground in sterile TNE buffer (100mM Tris-HCl, pH 8.0, 150mM NaCl, 10mM EDTA) containing 2mM aurointricarboxylic acid and filtered through Miracloth (Ostrem et al. 1987). SDS was added to the filtrate to 1% (w/v). The filtrate was mixed with an equal volume of phenol (500 g/200 mL water) containing 0.1% (w/v) 8-hydroxyquinoline. After 1-2 h at 4°C with occasional shaking the aqueous phase was removed and extracted twice with an equal volume of chloroform. Ammonium acetate (final conc. 0.3M) and an equal volume of isopropanol were added. After storing overnight at -20°C the pellet was dissolved in sterile DEPC-treated water. Total RNA was recovered by two precipitations in the presence of 2M LiCl. The RNA pellets were redissolved and precipitated again.

To extract RNA from spinach (*Spinacia oleracea*) 20 g of the tissue is rinsed with distilled H₂O, blotted dry,

minced with a razor blade, and then homogenized in 50 mL of extraction buffer (100mM Tris-HCl, pH 8.0, 100mM NaCl, 5mM EDTA, 1% N-lauryl sarcosine, 40 units heparin per mL) with a Polytron homogenizer for five 1-min. periods with 1 min. cooling periods on ice (Guy et al. 1985). This was followed by phenol/chloroform extractions and, later, washes of the RNA pellet with 3M sodium acetate, pH 5.5.

Leaves of maize (*Zea mays* L.) were ground with liquid N₂ and 25 g mixed with 100 mL 50mM Tris-HCl (pH 8.0) containing 10mM EDTA, 5M guanidinium thiocyanate, 2% (w/v) N-lauryl sarcosine, and 5% (v/v) 2-mercaptoethanol (Commere et al. 1986). The mixture was kept at 37°C for 15 min. and centrifuged at 20 000 rpm for 30 min. The pellet was treated as above in 50 mL of the same medium. Supernatants were pooled, adjusted to 0.1 mg mL⁻¹ CsCl and layered on 8 mL cushions of 5.7M CsCl, 50mM EDTA (pH 8.0) in 30 mL tubes. After centrifugation for 24 h at 25 000 rpm in a SW 27 rotor at 15°C, the RNA pellet was resuspended in 10mM Tris-HCl (pH 7.5) containing 1mM EDTA and 0.1% (w/v) SDS, and heated for 10 min. at 65°C. The RNA was precipitated with 0.1 vol. 3M sodium acetate (pH 6.0) and 2.2 vol. ethanol.

Tissues of germinating barley (*Hordeum vulgare*) were ground with liquid N₂ and added to 10 vol. 10mM Tris-HCl (pH 8.5) containing 5M guanidinium chloride, 5mM EGTA, 100mM mercaptoethanol, 0.1% lauryl sarcosine, and 0.001% octanol. The suspension was homogenized in a Sorvall Omnimixer at full speed for 2.5 min. Debris were removed by centrifugation and polymeric material precipitated with 0.75 vol. ethanol at -20°C for 16 h. The pellet was resuspended in 25mM trisodium citrate (pH 7.0) containing 5M guanidinium chloride, 5mM DTT, and 10mM EDTA. It was then precipitated with 0.5 vol. ethanol, dried, and redissolved in 100mM Tris-HCl (pH 9.0) containing 100mM NaCl and 2mM EDTA. Phenol (saturated with the same buffer)/chloroform extractions were done, and the RNA precipitated.

There are ways to extract RNA from as little as 100 mg of tissue (or cell cultures). This is thought to be sufficient for dot blot analysis and for Northern blot analysis (Davis et al. 1986). In one method the tissue is homogenized with a Polytron at medium speed for 15 sec. in the following extraction buffer: 100 µL 3M sodium acetate (pH 6), 20 µL 0.5M EDTA, 20 µL vanadyl ribonucleoside complex (BRL), 100 µL heparin solution (10 mg/mL), and 500 µL H₂O. To this is added 100 µL 10% SDS followed by vortexing. Several phenol/chloroform extractions are then done.

Recently there have become available (eg. Invitrogen, BRL) mRNA isolation kits to isolate intact poly(A)⁺ RNA directly from cells or tissue.

4 Preparation and characterization of poly(A)⁺ RNA (mRNA)

4.1 Characterization of mRNA

Messenger RNA (mRNA) represents about 1-4% of total RNA. Each mRNA codes for only one polypeptide and the sizes of mRNA molecules range from a few hundred to several thousand nucleotides long, depending on the size of the proteins for which they code. The enzyme RNA polymerase II is involved in transcribing the mRNA genes. Many plant mRNAs, except possibly chloroplast mRNAs, contain a 5' cap structure, a 5'→5' triphosphate linkage involving a 7-methylguanine residue that is added after transcription. The 5'-cap structure may play a part in the regulation of protein synthesis initiation. Most plant mRNAs have a poly-adenylic acid (poly-A) region at the 3' end which may be up to 250 nucleotides long. The "older" mRNAs may have shorter 3' poly-A tails. These are added after synthesis by a polyA-polymerase in the nucleus. However, unlike nuclear mRNAs and animal mitochondrial RNAs, plant mitochondrial transcripts do not contain poly-A tails (Clayton 1984). They can be retained on oligo (dT) columns, possibly due to internal A-rich regions. In studies with *Oenothera*, addition of an oligo A-tail to the 3' termini of the mitochondrial transcripts was necessary for the reverse transcriptase reaction (Hiesel and Brennicke 1987). In addition to the coding region, mRNAs contain untranslated nucleotides at either end that are of variable length. Some genes also have nucleotides that code for about 18-60 amino acids, at the N-terminus of a protein, that are not present in the mature protein. These amino acids are involved as signal or transit peptides. For example, over a hundred chloroplast proteins are first encoded in the nucleus, translated in the cytosol with transit peptides, and then transported through the chloroplast double membrane. Finally, the transit peptide is removed by a soluble protease in the chloroplast stroma.

Many mRNA transcripts are present as multigene families composed of two to 100 related but not identical genes. Such multigene families are probably formed by gene duplication and divergence through mutation. Some pseudogenes (incomplete genes) may also be present in the genome, as well as genes inactivated by insertion (transposable) elements. Genes, such as actin for lodgepole pine (*Pinus contorta*) (Kenny et al. 1988) may contain intervening sequences

(introns) that interrupt the amino acid coding regions. Introns usually contain the bases GU and AG at the left- and right-hand borders. After transcription the introns are cut out by processing enzymes in the nucleolus and the remaining coding sequences (exons) are ligated together. The number of introns varies with different genes; up to 50 may be present in some, with a size variation of from 10 to 1000 bp. Open reading frames may exist in some intron sequences.

4.2 Separation of m-RNA from r-RNA

Method 1: Oligo (dT) cellulose chromatography

Most eukaryotic mRNA molecules contain tracts of poly(A) at the 3' end and these can be up to 250 bases in length. Under high salt conditions (0.3-0.5M NaCl or KCl), poly(A) will hybridize to oligo (dT)-cellulose or poly(U)-sepharose. These are polymers of about 10-20 nucleotides covalently attached to a carbohydrate support, and binds RNA that may contain poly(A) tracts as short as 20 residues. Ribosomal and transfer RNAs do not possess poly(A) sequences and, therefore, will not bind. Following thorough washing of the column, mRNA can be recovered by eluting with a low salt buffer. However, with poly(U)-sepharose columns, the poly(A) binds more tightly and stronger elution conditions, such as the inclusion of formamide in the buffers, are required. The procedure includes an optional heat-treatment step to reduce aggregation of RNA prior to chromatography, and SDS is present in the buffers as a precaution against ribonuclease activity.

Materials

1. Binding and elution buffers as described in Appendix I.
2. Suspend 1.0 g of oligo (dT)-cellulose in 1X binding buffer and pipette the slurry into an 11 mL polypropylene disposable "Poly-Prep" chromatography column. Flush column with 20 mL 1X binding buffer and adjust flow rate to 10 mL/h. One g of oligo (dT)-cellulose will bind 20-40 A₂₆₀ units of poly(A).

Protocol

1. Centrifuge sample at 10 000 rpm for 10 min. and vacuum dry the pellet for a few minutes. Dissolve sample in 1 mL elution buffer (about 200 µg RNA/mL).

2. Heat the solution for 5 min. at 65°C, cool, add 1 mL of 2X binding buffer, and mix well. RNA is heated to disrupt any secondary structure that might form.
3. Load sample onto the column and, with 1X binding buffer, collect 24 mL of effluent.
4. Reapply the RNA which came through the column twice, after first heating to 65°C for 5 min. and then cooling. This is necessary to ensure that most of the poly(A)⁺ RNA will bind to the column. Discard this last effluent.
5. Wash column with 1X binding buffer.
6. The poly(A)⁺ RNA is removed with elution buffer, pH 7.6. Collect 1 mL and discard. Collect 12 mL poly(A)⁺ RNA.
7. Wash column with 1X binding buffer (about 3 volumes), then with 0.1N NaOH until strongly basic (2-3 volumes), and then again with 1X binding buffer (5-7 volumes).
8. Further purify poly(A)⁺ RNA from step 6 by repeating steps 2, 3, 5, and 6.
9. Add 50 µL/mL of 3M sodium acetate, pH 5.5, and then cold 95% ethanol. Place at -20°C at least 2 h.
10. Collect the RNA by centrifugation at 10 000 rpm for 10 min. and wash the pellet at least twice with 70% ethanol at room temperature to remove the SDS. Store as a precipitate under alcohol at -20°C (or lower).

Oligo (dT) Cellulose storage

1. Flush column with about 3 volumes of 1X binding buffer.
2. Flush column with 0.1M NaOH until strongly basic (2-3 volumes).
3. Flush column with 1X binding buffer and 0.02% sodium azide until pH 7.6 is reached (5-7 volumes).
4. Store column with this solution at 4°C. The column can be re-used indefinitely.

Note: The sodium salt of SDS is relatively insoluble and thus may tend to impede the flow of the column. This can be avoided by using lithium chloride instead of sodium chloride in the buffers. Further details of this method can be obtained by reference to Ausubel et al. (1989)

Method 2: Purification by Messenger Affinity Paper "Hybond - mAP" (Amersham)

This method is essentially as described by the manufacturers' instructions.

1. A piece of Hybond-mAP is cut to the required capacity, using scissors or scalpel while wearing gloves. The capacity is about 30-40 µg of mRNA per 1 cm². Usually 1 cm²/mg of total RNA is applied.
2. Wet mAP paper with 0.5M NaCl (autoclaved). Heat the RNA in distilled water at 60°C for 5 min. and quench on ice. Add NaCl to a final concentration of 0.5M NaCl. The RNA concentration should be about 1 mg/mL.
3. Place the Hybond-mAP paper on a few layers of filter paper (3 MM) cut to the exact size of the Hybond-mAP to insure that the RNA solution flows through the mAP paper. Add the RNA solution slowly.
4. Wash the mAP in three changes of 5 mL of 0.5M NaCl per 1 cm² for 10 minutes each wash. A rotary shaker can be used for this.
5. Rinse the Hybond-mAP in 70% (v/v) ethanol for 2 min., then blot dry on 3 MM paper.
6. Transfer the Hybond-mAP to the minimum volume of sterile double distilled water required to cover the paper and incubate at 70°C in a water bath for 5 min..
7. Remove the mAP using clean, sterile forceps. The poly(A)⁺ RNA remains in the water. A second round of isolation should be performed by adjusting the NaCl concentration in the released mRNA to 0.5M and respotting on a new piece of pre-wet Hybond-mAP paper.

4.3 Electrophoresis of m-RNA with agarose gels

RNA extracts are routinely analyzed by electrophoresis using agarose gels containing formaldehyde. This is done to determine the quantity of contaminating ribosomal (r) RNA and transfer (t) RNA present in the extracts. However, even after two passes of m-RNA through the oligo (dT)-cellulose columns, some r-RNA will still be present. Even so, the sharpness of these bands will indicate if mRNA is degraded to some extent. Plant RNAs of 25S and 18S rRNA should appear as thin bands, while mRNA [poly(A)⁺ RNA] will appear as a smear after staining the gels with ethidium bromide, or of the nylon membranes after Northern transfer with methylene blue.

Methods:

The following method is essentially as described by Fournier et al. (1988).

Buffers

10X MOPS/EDTA buffer:
 0.2M MOPS [3-(N-morpholino)
 propanesulfonic acid]
 50mM sodium acetate
 10mM EDTA
 Adjust to pH 7.0 and autoclave
 Electrophoresis sample buffer:
 0.75 mL deionized formamide
 0.15 mL 10X MOPS
 0.24 mL formaldehyde (37%)
 0.1 mL deionized RNase-free H₂O
 0.1 mL glycerol
 0.08 mL 10% (w/v) bromphenol blue
 Prepare fresh or store at -20°C in small
 aliquots
 Electrophoresis buffer:
 1X MOPS/EDTA buffer

Sample preparation

1. Dissolve RNA in 25 mM EDTA, 0.1% SDS
2. Add 1-3 µg poly(A)⁺ RNA or 10-30 µg total RNA to a microcentrifuge tube
3. Adjust volume to 5 µL with DEPC-treated, autoclaved water.
4. Add 25 µL Electrophoresis Sample buffer and heat at 65°C for 15 min. to denature RNA.

5. Add 1 µL ethidium bromide solution (1 mg/mL in deionized RNase-free H₂O).
6. Load sample on the gel. Markers (eg., BRL's 0.16-1.77 Kb RNA ladder) can be added to the gel.

Gel preparation and electrophoresis

1. Add 1-1.5 g agarose, 10 mL 10X MOPS, and 87 mL DEPC-treated autoclaved H₂O to an RNase-free flask.
2. Dissolve agarose and let cool to 50°C.
3. In a fume hood, add 5.1 mL 37% formaldehyde into the agarose solution, mix gently, and pour into a gel tray.
4. Allow the gel to sit for 1 h before use.
5. Prior to loading the gel, flush sample wells by pipetting electrophoresis buffer in and out of the wells.
6. Load wells and electrophoresce at 30V (constant voltage) at room temperature for 18 h. Higher voltages have been used by others, eg. 100V for 2-3 h. The dye migrates ~10 cm into the gel.
7. For 10 µg of total RNA photograph at 302 nm or 254 nm using a yellow filter (eg. Kodak Wratten #9) and Polaroid type 665 PN film for 4 sec. at f4.5.

4.4 Transfer preparation: Northern blotting

1. Soak gel for two 20 min. periods in 500 mL of 10X SSC at room temperature shaking gently. If a thick gel or high conc. of agarose (>1.3%) is used, soak gel for 10-20 min. in 0.05M NaOH in 1X SSC.
2. Prewet the nylon membrane in distilled water for 5 min., followed by a 5 min. soak in 10X SSC.
3. Transfer the RNA (~ 8 h) in 10X SSC using a sponge (with small pores or one made from polyurethane foam) placed in the solution to enhance capillary action. Subsequent layers include 3 layers of 3MM paper; the gel; the nylon membrane; 3 layers of 3MM paper; 3-4 cm of paper towels; a glass plate; and a weight of 0.2-0.4 kg.

4. Fix the RNA to the nylon membrane by baking for 2 h at 80°C or by placing the membrane, with the RNA-side down, on a shortwave (254 nm or 302 nm) transilluminator for 5 min. The RNA bound to the membrane can be viewed for transfer efficiency or photographed under uv transillumination; no staining is required. After baking, the filter can be stored at room temperature for weeks. If stored, the filter should be rebaked before prehybridization.

Hybridization and autoradiography

1. Prehybridize (1-4 h) and hybridize (12-36 h) according to methods provided by the supplier of the membrane.
2. Wash membranes under moderate stringency: two 20 min. washes in 1X SSC, 0.1% (w/v) SDS at room temperature, followed by two 20 min. washes in 0.1X SSC, 0.1% SDS at 50-55°C.
3. Autoradiograph with Kodak XAR film at -70°C.

4.5 Size fractionation of mRNA

Sucrose gradient separation (eg. Casey et al. 1985):

1. Place the poly(A)⁺ RNA (70-100 µg) at 65°C for 5 min. in 100 µL of 10% (v/v) DMSO, 10mM Tris-HCl (pH 7.5), 10mM LiCl, and 1mM EDTA.
2. Rapidly cool on ice and then layer on a 10 mL linear 5-20% (w/v) sucrose gradient in 95% (v/v) DMSO, 4% (v/v) formamide, 10mM Tris-HCl (pH 7.5), 10mM LiCl, and 1mM EDTA.
3. Centrifuge for 48 h at 150 000 rpm and 25°C.
4. Recover the RNA from 200 µL fractions of the sucrose gradient tube by ethanol precipitation at -20°C with 0.15M sodium acetate (pH 4.8) and 3 µg calf liver tRNA per fraction.
5. Wash RNA precipitate with 80% ethanol, dry, and then dissolve in water (10 µL/fraction).

Casey et al. (1985) used 0.5 mL redissolved RNA from each gradient fraction for *in vitro* translation studies to determine the location in the fractions of the mRNA that encoded specific seed proteins.

5 Preparation and characterization of cDNA

5.1 Methods of preparing cDNA

Kimmel and Berger (1987) have described several different approaches to construct cDNA from mRNA. The basic requirements for first-strand synthesis include mRNA, a complementary primer, reverse transcriptase, and deoxyribonucleoside triphosphates. The primer can be oligo (dT), random hexamers, or specific oligomers. Oligo (dT) is usually employed for cloning because it binds to the 3' poly(A) tail of mRNA and is more efficient at making full-length clones. Using random hexamers result in equal representation of the mRNA sequence, but full-length cDNAs are not achieved. Specific oligomers bind to known sequences, and transcription is only to one or to a few mRNAs. This primer extension procedure results in fragmentation.

For second-strand synthesis, one procedure is to nick the RNA in the RNA-DNA hybrids with *E. coli* RNase H and use the fragments to make cDNA strands in the presence of DNA polymerase I. In the classical method, a hairpin loop occurring at the 3' end of the newly synthesized first strand serves as a primer to make the second strand in the presence of dNTPs, the Klenow fragment, and reverse transcriptase. The hairpin loop is cleaved with a single-strand-specific nuclease such as mung bean nuclease or S₁.

In order to clone the final cDNA insert to a plasmid or bacteriophage DNA, the cDNA can be tailed and annealed to a cleaved plasmid having complementary homopolymeric tails. Alternatively, linkers or adaptors can be attached to the blunt-ended cDNA insert. cDNA produced by the RNA-DNA hybrid method can be incubated with terminal transferase and dGTP and then ligated to the tailed plasmid. This is a simple and efficient method of making cDNA libraries of up to 50 000 clones. The original pBR322 vector has been replaced with newer ones such as the pUC plasmids. If the classical method is used to produce cDNA, cloning by tailing to a tailed plasmid is not recommended due to problems associated with mung bean and S₁ nuclease. These nucleases can eliminate unpaired sequences at the hairpin and produce nicks in the cDNA, resulting in problems with cloning due to the formation of branched structures by terminal transferase.

Linkers and adaptors can also be used to attach cDNA to the vector. It is better to use high concentrations of linkers because that promotes the ligation reaction of cDNAs and it also inhibits self-ligation of

inserts. This procedure requires that the cDNA be first methylated to prevent cleavage by the restriction endonuclease used to create the staggered ends of the cDNA for ligation to the vector (eg. λ gt10, λ gt11). For direction cloning, cDNA produced by the classical method can be used, although the cDNAs would not be full-length. In this method, one type of linker is attached to the blunt end of the cDNA, the hairpin loop is cleaved, and the second type of linker is added to both termini of the final double-stranded cDNA. Two different types of restriction endonucleases are then used to cleave the two types of linkers at either end. A newer method for directional cloning has been introduced by Promega. This involves the use of an *Xba*I primer-adaptor (5' GTCGACTCTAGA(dT)₁₅3') which hybridizes to the poly-A tail of the mRNA. After first- and second-strand cDNA synthesis (using RNase to produce nicks in the RNA-DNA hybrid) *Eco*RI linkers are added to both ends of the cDNA insert. Following *Eco*RI and *Xba*I digestion, the inserts can then be directionally cloned into a λ vector having unique *Eco*RI and *Xba*I cloning sites. We are now using this procedure to construct subtraction libraries to try to isolate stage- and tissue-specific genes during the germination of jack pine seeds. Some of these procedures are discussed in more detail in Section 7.

When screening with nucleic acid probes, and constructing large cDNA libraries, vectors such as λ gt10 or λ ZAP could be used. The cDNA should be constructed using RNase H to produce nicks in the RNA-DNA hybrid, followed by the use of linkers to clone into a suitable vector. Generating a library of $>10^6$ clones may require 10-40 μ g of mRNA. With respect to low-abundance mRNAs, Maniatis et al. (1982) presented examples of the number of clones needed to ensure their presence.

Vector-primed cDNA synthesis has some advantages even though the procedure may be more difficult and time consuming than traditional cDNA cloning protocols (eg. Okayama and Berg 1982). Some of the advantages are the relatively low backgrounds of clones containing no inserts, the high yields of cDNA clones obtained from small amounts of mRNA, and the ability to prepare long cDNAs that have a high proportion of full-length clones. But there are some disadvantages: though the number of full-length molecules are enhanced, total clone production is reduced and, as this method requires a threefold molar excess of mRNA relative to the vector primer, it is highly inefficient. Protocols for vector-primed cDNA synthesis, such as that using pUC18 or pARC as vectors, are described by Deininger (1987). There are a variety of methods and vectors available for this type of cDNA cloning. For example, the dimerized pARC7

vector eliminates certain steps of the original method of Okayama and Berg (1982) and gives two vector molecules attached to separate cDNAs. Specialized vectors with multilinker cloning sites are usually used, such as those capable of producing large quantities of RNA from the cDNAs *in vitro* with specific RNA polymerases (eg. SP6). These can be used for *in vitro* translation reactions followed by immunological characterization.

5.2 Some factors affecting first-strand cDNA synthesis

For first strand synthesis, a buffer with pH of 8.3 (Tris is best) is optimal for efficient incorporation and production of full-length transcripts (eg. Maniatis et al. 1982). A deviation of ± 0.5 pH units is thought to result in a five-fold decrease in the production of full-length transcripts. For AMV reverse transcriptase, the pH should be adjusted at 42°C. Optimal potassium ion concentration for both total synthesis and length of cDNA is 140-150mM. Sodium ions result in smaller transcripts. Divalent cations are an absolute requirement, with the optimal concentration for Mg^{++} being 6-10mM.

High concentrations of the four deoxyribonucleoside triphosphates (dNTPs) are important for increased yields of full-length transcripts. Usually, concentrations of 200-250 μ M are used. The dNTPs should be dissolved at a high concentration (>40 mM) and diluted to stock solutions of 10mM each. The diluted solutions should not be stored. A mixture should be made of each and then filter-sterilized. Molar extinction coefficients of the bases and wavelength of maximum absorption are, for a 1-cm path length: 1.54 $\times 10^4$ (259 nm), adenine; 9.1 $\times 10^3$ (271 nm), cytosine; 1.37 $\times 10^4$ (253 nm), guanine; 1.0 $\times 10^4$ (262 nm), uracil; and 7.4 $\times 10^3$ (260 nm), thymine.

A concentrated solution (eg. 100 μ g/mL) of the primer oligo 12-18 (dT) is prepared in 0.1% diethyl pyrocarbonate, followed by stirring at room temperature and then heating at 70°C for 1 h. A solution of 80mM sodium pyrophosphate is prepared as for oligo (dT). Both pyrophosphate and actinomycin D inhibit second-strand synthesis and, with the AMV enzyme, the yield of full-length cDNA is enhanced with the former.

Pyrophosphate should not be used with the mouse Moloney leukemia virus (MMLV) enzyme (Krug and Berger 1987). Instead, actinomycin D is required. Spermidine is included in some protocols with the AMV enzyme as it is thought to increase the

yield of full-length cDNA. A ribonuclease inhibitor, such as RNasin, is included in the reaction because it can sometimes increase the yield of cDNA.

Before use, poly(A)⁺ RNA should be heated to 70°C for 3-5 min., followed by cooling immediately before use. Methylmercury hydroxide is sometimes used to eliminate extensive secondary structure of the RNA, although this can decrease the yield of cDNA.

Reverse transcriptase has *in vitro* both a polymerase activity and an associated ribonuclease H (RNase H) activity. To produce a DNA strand complementary to the RNA, the polymerase requires a template RNA molecule hybridized to a DNA primer with a 3'-hydroxyl group and four deoxyribonucleoside triphosphates. However, during this process we also find RNase H, an exonuclease that degrades RNA in DNA-RNA hybrids, (Krug and Berger 1987). Various chemicals eg., pyrophosphate, fluoride, RNasin, have been tried to inhibit the ribonuclease activity of reverse transcriptase from avian myeloblastosis virus (AMV), but without much success. Some preparations of reverse transcriptase, however, may differ in the ratio of the two activities. In addition to the AMV reverse transcriptase, a cloned MMLV reverse transcriptase is available. High concentrations of reverse transcriptase (dNTPs) promote the synthesis of full-length cDNAs. Maniatis et al. (1982) has suggested that high yield and increased size of the cDNA transcripts may require up to 80 units of enzyme per mg of template. Reverse transcriptases should be stored at -70°C in small aliquots.

5.3 First strand cDNA synthesis reaction

Stock reagents:

AMV reverse transcriptase 5X buffer:	
250mM Tris-HCl, pH 8.3	
750mM KCl	
50mM MgCl ₂	
5mM dithiothreitol	
5mM EDTA	
50 µg/mL bovine serum albumin	
sodium pyrophosphate	80mM
human placental	
ribonuclease inhibitor	20 units/µL
deoxyribonucleoside	
triphosphate mix	10mM dATP, dGTP, dTTP; 5mM dCTP
oligo dT primer	
(12-18)	2 µg/µL
or	
random hexanucleotide	
primer	150 ng/µL
or	

primer-adaptor	
(Xba, EcoRI)	
(Promega)	1 µg/µL
reverse transcriptase	20 units/µL

The components in the reaction mix can vary in terms of concentration and type, depending on the supplier (eg., Amersham, BRL, and NEM). At present we are using some parts of the kit from Amersham. Recently Invitrogen has introduced a cDNA synthesis and library construction kit, which it claims has some advantages over the other ones. Phagemid vectors are used.

The following reaction mix is for 1 or 5 µg of poly(A)⁺ mRNA. The enzymes should be kept at -20°C until just before use, while the other components should be kept on ice following thawing from storage at -20°C. The reaction should be in a sterile 1.5 mL microcentrifuge tube on ice. Add the following components in order:

1 µg	or	5 µg	
4 µL		10 µL	5X first strand synthesis reaction buffer
1 µL		2.5 µL	sodium pyrophosphate solution
1 µL		2.5 µL	human placental ribonuclease inhibitor
2 µL		5 µL	deoxynucleoside triphosphate mix
1 µL		2.5 µL	oligo (dT) ₁₂₋₁₈ primer (or 0.5 µg/µg RNA primer-adaptor or 2 or 5 µL random hexanucleotide primer)
X µL		X µL	5 µCi[α- ³² P]dCTP (optional)
Y µL		Y µL	mRNA (can be heated at 60°C for a few minutes prior to reaction)
Z µL		Z µL	H ₂ O
To a volume of 20 µL or 50 µL for 1 µg or 5 µg mRNA, respectively.			

- Mix gently and spin for a few sec. in a microcentrifuge
- Add 20 units of reverse transcriptase per µg mRNA

- Incubate at 42°C for 1-2 h and then put on ice
- Remove a sample to determine efficiency of labelling (see Section 5.5).

5.4 Second-strand cDNA synthesis

After completion of the first-strand synthesis reaction, the RNA-DNA hybrid is left intact. The RNA is nicked with RNase H, leaving only small RNA primers with free 3'-OH groups attached to the cDNA. These 3'-OH groups are then used by DNA polymerase I to synthesize a second strand along the length of the first strand. Before cloning at *EcoRI* sites of λ DNA, T4DNA polymerase is used to create blunt ends to assure efficient ligation of the linkers to the cDNA inserts. Although actinomycin D or sodium pyrophosphate and high mRNA template are used in the first strand reaction to suppress second-strand synthesis by reverse transcriptase, loops at the 3' ends of the first strand can remain. These loops can be used by DNA polymerase I as primers for second-strand synthesis, resulting in cDNAs that cannot be cloned. Methods to determine the presence and extent of such loops are described by Gubler (1987). For example, the hairpin loops can be cleaved with mung bean nuclease or S_1 nuclease.

Stock reagents:

Reaction buffer, 2X	
40mM Tris-HCl, pH 7.5	
10mM MgCl ₂	
20mM (NH ₄) ₂ SO ₄	
200mM KCl	
100 μ g/mL bovine serum albumin	
<i>E. coli</i> ribonuclease H	0.8 units/ μ L
<i>E. coli</i> DNA polymerase I	6 units/ μ L
T4 DNA polymerase	4.5 units/ μ L

Second strand reaction mixture

To the first strand cDNA synthesis mix, on ice, add the following components in order:

1 μ g original or 5 μ g original

mRNA	mRNA	
37.5 μ L	93.5 μ L	Second strand synthesis reaction buffer
A μ L	A μ L	50 μ Ci [α - ³² P] dCTP (optional)

0.8 units	4.0 units	<i>E. coli</i> ribonuclease H
23 units	115 units	<i>E. coli</i> DNA polymerase I
B μ L	B μ L	H ₂ O

To a final volume (including enzyme) of 100 μ L and 250 μ L for the 1 μ g and 5 μ g mRNA samples, respectively.

- Mix gently and incubate at 12°C for 60 min. and then at 22°C for 60 min.
- Incubate at 70°C for 10 min.
- Spin for a few sec. in a microcentrifuge
- Place on ice and add 2 units of T4DNA polymerase per microgram of original mRNA
- Mix gently and incubate at 37°C for 10 min.
- Stop reaction by adding 4 μ L of 0.25M EDTA (pH 8.0) per 100 μ L of final reaction mix
- Remove a sample to test isotope incorporation

5.5 Calculation of percentage incorporation

To monitor the reaction of cDNA synthesis one needs to determine the amount of radioactivity on dry filters, wash the filters to remove unincorporated labelled substrate, and to count the identical filters again. There are three ways to count ³²P on filters. One instrument by Bioscan (Model QC2000) responds to β particles and measures ³²P with a counting efficiency of ~30%. The Bioscan counter measures charged particles and not Cerenkov radiation. Cerenkov radiation occurs within the glass or plastic wall of a scintillation vial when a dry filter is placed flat on the bottom of the vial. Radioactivity is determined with the ³H channel of a scintillation counter, and a counting efficiency with ³²P is ~25%. In the third method, a counting efficiency of almost 100% is obtained when using a scintillation fluid and the ³²P channel. We are routinely using the Bioscan as the unit is inexpensive and takes up little bench space.

1. Add 5 μ Ci of [α -³²P] dCTP to the first strand synthesis reaction mix (20 μ L).
2. After first strand cDNA synthesis, remove a 1 μ L sample and process as below.

3. For the second strand synthesis reaction mix use 50 μ Ci of [α - ^{32} P] dCTP. After the reaction, remove 1 μ L and proceed as below.
4. Transfer a 1-2 μ L aliquot of the above reaction mixture to a tube with 20 μ L water or 0.2M EDTA and mix well.
5. Spot 5 μ L onto the centre of each of two 2.4 cm Whatman DE81 paper discs. For the first strand mix label these A and B, and then C and D for the second strand mix.
6. Wash filters B and D six times, with 5 min. per wash in 0.5M Na₂HPO₄ followed by two washes in water (1 min. per wash) and two washes in 95% ethanol (1 min. per wash). Filters B and D measure CPM incorporated into the cDNA.
7. Dry both filters (eg. with infra-red lamp) and count.

Percentage incorporation of the first strand reaction is:

$$\frac{\text{CPM on filter B}}{\text{CPM on filter A}} \times 100\%$$

To calculate percentage incorporation during second strand synthesis, the amount of radioactivity incorporated into the first strand reaction is first subtracted. Values A and B are first divided by 6, as the first strand reaction mix is 1/6th the volume of the second strand mix. Thus percentage incorporation during second strand synthesis is:

$$\frac{D-B/6}{C-A/6} \times 100\%$$

To calculate amount of cDNA synthesized for the 20 μ L first strand and 100 μ L second strand reaction mixes the formula is:

$$(\% \text{ incorporation} \times 140) \text{ ng}$$

To calculate the percentage of mRNA transcribed the formula is:

$$\frac{\text{Amount of first strand cDNA synthesized}}{\text{Amount of input mRNA}} \times 100\%$$

To estimate success of second strand synthesis the formula is:

$$\frac{\text{Amount of second strand cDNA}}{\text{Amount of first strand cDNA}} \times 100\%$$

5.6 Purification of cDNA

1. Add 250 μ L (or equal volume) phenol/chloroform (saturated with TE)
2. Vortex and spin for 1 min.
3. Remove the upper aqueous phase and repeat phenol/chloroform extractions
4. Add two vol. of chloroform to the aqueous phase and mix well
5. Spin briefly and save the upper phase

Removal of unincorporated nucleotides

These can be removed by Sephadex G50 chromatography or by ethanol precipitation with ammonium acetate.

A. Ethanol precipitation with ammonium acetate

To the phenol extracted cDNA add:

1. An equal vol. of 4M ammonium acetate
2. Add twice the combined vol. of cold (-20°C) ethanol
3. Chill for 15 min. on dry ice or suitable freezer (-70°C)
4. Warm to RT with gentle shaking
5. Spin for 10 min.
6. Remove supernatant, being careful not to disturb the pellet
7. Wash the pellet once in 50 μ L 2M ammonium acetate, 100 μ L (-20°C) ethanol, at RT by gently shaking, centrifugation, and aspiration

8. To the pellet add 200 μ L cold (-20°C) ethanol, being careful not to disturb the pellet
9. Spin for 2 min.
10. Carefully remove supernatant
11. Dry pellet in a vacuum desiccator for a maximum of 2-3 min.
12. Resuspend in a suitable vol. of TE
13. Store at -20°C

B. Chromatography through Sephadex G50

When cDNA is synthesized using 5 μ g mRNA the material should be divided in two and applied to separate Sephadex G-50 columns. After recovery from the columns cDNA is pooled prior to ethanol precipitation.

1. Plug the bottom of a 1 mL disposable syringe with a small amount of sterile glass wool. In the syringe prepare a column (0.9 mL bed vol.) of Sephadex G50 equilibrated in buffer
2. Insert syringe into a centrifuge tube and centrifuge at 1600 rpm for 4 min. Continue to add Sephadex until the packed column volume is 0.9 mL
3. Add 0.1 mL TE, 0.1M NaCl and re-centrifuge as above
4. Repeat step 3 until the vol. eluted is $\sim$ 0.1 mL. Transfer syringe to a fresh centrifuge tube
5. Apply cDNA to the column in a total vol. of 0.1 mL (use Sephadex G50 buffer to make up the vol.)
6. Centrifuge as in step 2, collecting the 0.1 mL of eluate from the syringe. The unincorporated nucleotides remain in the syringe and should be carefully discarded (radioactive material)
7. To the cDNA add 0.1 vol. of 3M Na acetate and 2 vol. ethanol (-20°C). Place in dry ice

and ethanol bath for 20 min. Centrifuge for 15 min.

8. Remove the supernatant, being careful not to disturb the DNA pellet
10. Centrifuge for 2 min. in a microcentrifuge
11. Carefully remove supernatant
12. Dry pellet in a vacuum desiccator for 2-3 min.
13. Resuspend cDNA in TE buffer and store at -20°C .

Note: Sephadex G-50 pre-made columns can be purchased as "Nick Columns" from Pharmacia.

Gel electrophoresis of cDNA

Preparation of sample

1. Remove a sample from the cDNA synthesis reaction mix that represents 10 000-30 000 CPM of radioactivity. Place in a microcentrifuge tube and proceed to remove any remaining RNA.
2. Add 20 μ L of carrier DNA solution (100 μ g/mL salmon sperm DNA in 20mM EDTA)
3. Add 1/3 of the combined volume with 1M NaOH
4. Incubate at 46°C for 30 min.
5. Spin for a few sec. in a microcentrifuge and add a volume of 1M HCl equal to the volume of NaOH added in step 3.
6. Add a vol. of 1M Tris pH 8.0 equal to that of the vol. of 1M HCl added in step 5.
7. The resulting mixture should be purified with phenol/chloroform extractions and ethanol precipitations, as described earlier for the purification of cDNA
8. Resuspend pellet in a suitable vol. of alkaline agarose gel loading buffer:

50mM NaOH
1mM EDTA
2.5% Ficoll (Type 400 Pharmacia)
0.025% bromocresol green (or freshly
prepared bromphenol blue).

Electrophoresis

1. To obtain a 1.4% gel add agarose to 50mM NaCl and 1mM EDTA (prepare a 10X stock of buffer)
2. Heat in a microwave until the agarose dissolves.
3. Cool solution to 50°C and pour into the gel tank.
4. Add alkaline electrophoresis buffer to cover the gel to a depth of 3-5 mm: A 10X stock is 300mM NaOH, 10mM EDTA
5. Allow buffer to soak into the gel for at least 30 min. before loading the DNA. During electrophoresis the buffer should cover the gel to a depth of 1 mm.
6. Load the cDNA sample and allow electrophoresis at voltages up to 7.5 v/cm until the dye has migrated approximately 1/3 the length of the gel. As bromocresol green may diffuse from the gel after it has migrated out of the loading slot, the power should be turned off and a glass plate placed on the gel before continuing electrophoresis.
7. Remove gel and soak for 30 min. at RT in 7% trichloroacetic acid (two changes).
8. Mount gel onto a glass plate and dry for several hours under many layers of Whatman 3MM paper weighted with another glass plate. The dried gel is covered with plastic wrap before autoradiography at -70°C.

5.7 Size-fractionation of cDNA

5.7.1 Sepharose CL-4B chromatography of cDNA

The following method is essentially as described by Eschenfeldt and Berger (1987).

1. A column of 20X 1 cm (diameter) is made (eg. Econo column of Bio-Rad) with a slurry of Sepharose CL-4B in water until a bed height of

19 cm is reached. The column is run and stored at RT.

2. Wash with water, followed by several column volumes of water saturated with 0.05% diethyl pyrocarbonate.
3. Wash with water to remove the diethyl pyrocarbonate and equilibrate the column with 50mM NH_4HCO_3 , pH 8.
4. Calibrate column using 100 μL HaeIII-digested ϕX174 DNA at 20 $\mu\text{g}/\text{mL}$. Upon loading the sample, collect 0.20 to 0.25-mL fractions (7 drops) in a fraction collector at a pressure of 30-35 cm. Transfer fractions to 1.5 mL Eppendorf tubes and reduce to dryness. Analyze each entire fraction in a 2% agarose gel. cDNA of 600 bp and larger are found in the void volume, while smaller molecules appear after about 5.5 mL of effluent have been collected.
5. Dissolve sample cDNA in 100 μL water. Labelled material is usually not required if the calibration was done carefully. Load the sample and activate the fraction collector. When sample has entered the gel, wash with 100 μL of buffer, connect the reservoir of NH_4HCO_3 , and collect fractions as above.
6. Pool the high molecular weight cDNA fractions on the ascending front and 2-3 fractions on the descending limb front.
7. Reduce pooled material to dryness, rehydrate with 0.5 mL water, and then dry again. Dissolve in 20 μL water, add 5 μg poly(A)⁺ as carrier, if necessary, and ethanol precipitate using potassium acetate as the salt (3M, pH 5.5). The sample can be reprecipitated a second time to remove residual NH_4HCO_3 (terminal transferase and T4 polynucleotide kinase are very sensitive to ammonium ions).

Sepharose CL-4B columns may be regenerated and reused for years.

As described by Maniatis et al. (1982), the cDNA is first dissolved in 10 μL H_2O and to that is added 10 μL 20mM Tris-HCl (pH 8), 0.6M NaCl, and 2mM EDTA. The cDNA is fractionated into size classes by passage through a 1 mL column of Sepharose CL-4B equilibrated in the same buffer. Fractions of 50 μL are collected and an aliquot of each assayed by alkaline agarose gel (1.4%) electrophoresis using as MW

markers a set of end-labelled fragments of pBR322 DNA. The position of the cDNA is located by autoradiography, and fractions of cDNA greater than 500 bp are collected and precipitated with ethanol.

5.7.2 Use of low melting agarose

1. Make an agarose gel (0.7-1.5%) using TBE buffer and containing ethidium bromide (0.5 µg/mL).
2. Dissolve agarose, cool to 50°C, and pour into the gel tank containing TBE buffer with ethidium bromide.
3. Load the cDNA samples together with MW markers, eg. λ /HindIII.
4. Electrophorese at 25-30 mA at 4°C.
5. Cut out the appropriate cDNA fraction and put in a microcentrifuge tube. Add 3-5 vol. 20mM Tris-HCl (pH 7.5), 1mM EDTA, and put at 65°C for 10 min.
6. Add an equal volume of phenol, vortex, and spin for 10 min.
7. Remove and save the upper, aqueous phase in a fresh tube.
8. To the lower, phenol phase, add 0.5 vol. 20mM Tris-HCl (pH 7.5) and 1mM EDTA. Vortex and spin for 10 min.
9. Remove the upper, aqueous phase and combine with the aqueous phase retained in step 7.
10. Repeat steps 6-9.
11. To the final pooled aqueous phase, add an equal volume of chloroform/isoamyl alcohol (24:1). Vortex and spin for 2 min.
12. Remove the upper, aqueous phase into a fresh tube and add 0.1 vol. 3M sodium acetate and 2 vol. ethanol (-20°C). Place at -70°C for 20 min. and centrifuge for 15 min.
13. To the pellet add 200 µL (-20°C) ethanol and centrifuge 2 min.

14. Remove supernatant and dry the pellet in a vacuum desiccator for 2-3 min. Resuspend pellet in TE buffer and store at -20°C.

5.7.3 Separation of cDNA on polyacrylamide gels

A method was described by Davis et al. (1986) to remove excess *Eco*RI linkers and small cDNA fragments. The cDNA is size-fractionated on a 5% polyacrylamide gel run in TBE buffer. The sample is run in parallel with size markers which include pBR322 digested with *Hinf*I and *Eco*RI and end-labelled with the large fragment DNA polymerase I and ^{32} P-dATP, as follows:

1-2 µg pBR322 DNA	10 µL
10X <i>Eco</i> RI buffer	2 µL
α - ^{32} P-dATP	4 µL
<i>Hinf</i> I (10 U/µL)	1 µL
<i>Eco</i> RI (10 U/µL)	1 µL
H ₂ O	2 µL

Incubate at 37°C for 60 min. Add 1 µL of DNA polymerase I (large fragment, 5 U/µL) and incubate 15 min. at 37°C. Heat sample to 65°C for 10 min. and remove unincorporated ^{32} P-dATP via Elutip purification of the digest. MW markers are 998, 631, 517, 506, 396, 344, 298, 221, 220, 154, and 75 bp. After 2-3 h of electrophoresis at ~10V/cm all cDNA migrating at over 500 bp is excised and the cDNA removed by electroelution. Place sample in a dialysis bag clipped at each end, with 1 mL 1XTBE buffer and 10 µg/mL yeast tRNA, and elute for 12-16 h. The cDNA can be concentrated by passing through an Elutip-d minicolumn (Schleicher and Schuell, Inc.). The cDNA is then ethanol precipitated.

5.7.4 Separation of cDNA on sucrose gradients

Below is a brief description of the method of Bartels and Thompson (1983).

1. Size-fractionate in 4.5 mL gradients using a 5-20% sucrose gradient in:

10mM Tris-HCl, pH 8.0
1mM EDTA
10mM NaCl

at 50 000 rpm with a SW 65 rotor (Beckman) for 4 h at RT.

2. Collect 20 fractions

3. Analyze a small aliquot of each fraction on a 5% polyacrylamide-urea gel to estimate size of cDNA in the presence of 10mM Mg-acetate.
4. Ethanol precipitate in the presence of 10mM Mg-acetate cDNA fractions containing cDNA bigger than about 500 nucleotides.

6 cDNA cloning

6.1 cDNA cloning with bacteriophage λ

6.1.1 Preparation of *EcoRI* linked cDNA

There are various protocols and kits available to clone cDNA molecules. At present we are using part of the kit from Amersham to obtain *EcoRI*-linked cDNA, followed by ligation to λ ZAP (Stratagene), and *in vitro* packaging (Stratagene). With the use of linkers, one requires sequential methylation of the insert, ligation of phosphorylated linkers to the cDNA, *EcoRI* digestion of linked cDNA, and removal of excess linker molecules such as by Sepharose CL-4B column chromatography as described earlier or by prepared columns such as available from Amersham.

Methylation of cDNA

Internal *EcoRI* sites of the cDNA are protected by first methylating with *EcoRI* methylase. Using a 1.5 mL Eppendorf tube, between 0.5 and 1 μ g of DNA is methylated in 20 μ L DNA methylase buffer with 20 units of *EcoRI* methylase. DNA methylase buffer is:

50mM Tris-HCl (pH 8.0)
100mM NaCl
10mM EDTA
0.2 mg/mL BSA
0.1mM S-adenosylmethionine

With the Amersham kit all cDNA cloning components (except for the columns and *in vitro* packaging extracts) are kept at -20°C . Also the S-adenosylmethionine is supplied as a 100X stock solution and kept separate from the rest of the above DNA methylase buffer until just before use. The reaction mix is kept at 37°C for 60-90 min., followed by 70°C for 10 min. to inactivate the *EcoRI* methylase. In some protocols, this mixture is first extracted with phenol-chloroform and precipitated before the next step. With the Amersham system, the methylation, addition of *EcoRI* linkers, and digestion with *EcoRI* is done in the same tube.

Addition of *EcoRI* linkers to cDNA

EcoRI linkers [5'd(GGAATTCC)] can be obtained that are already phosphorylated at the 5' end. Methods to phosphorylate linkers are available, eg., Wu et al. (1987). Also required are T4 DNA ligase (5 units per 30 μ L reaction volume) and T4 DNA ligase buffer (see Appendix I; usually kept as a 10X stock). With the Amersham protocol, 1 μ L of *EcoRI* linkers are used with 1 μ g of cDNA in a total volume of 30 μ L. To the cDNA is added in order; the ligase buffer, *EcoRI* linkers, water, and the T4 DNA ligase. This reaction mix is then mixed gently and placed at 15°C for 16-20 h. The reaction is stopped by heating at 70°C for 10 min. to inactivate the ligase, and kept at -20°C if not proceeding to the next step. According to Wu et al. (1987) 1mM hexaminecobalt chloride stimulated ligation, and was used in their reactions.

EcoRI digestion of linked cDNA

To the 30 μ L linked cDNA reaction mix is first added *EcoRI* buffer (10X conc.), 10 μ L; water (to a final volume of 100 μ L); and 100 units of *EcoRI*. After mixing, this is placed at 37°C for at least 5 h and heated to 70°C for 10 min. to stop the reaction. *EcoRI* digestion buffer is:

100mM Tris-HCl (pH 7.5)
50mM NaCl
10mM MgCl₂
0.1 mg/mL BSA

Some suppliers (eg. BRL) include digestion buffers with purchase of the enzyme.

As described earlier, the unwanted free linkers are removed and the linked cDNA is precipitated by adding 1/10th volume 3M sodium acetate and 2.5 volumes of ethanol. After leaving at -20°C for at least 2 h, the cDNA pellet is dried and resuspended in buffer (eg. STE) for ligation to the vector.

6.1.2 Ligation of cDNA to λ ZAP and λ gt10 vector arms

For λ gt10 vector

Reaction mix:

Linked insert cDNA 100-500 ng
 λ gt10 arms 1 μ g

10X T ₄ DNA ligase	
buffer	1 μ L
H ₂ O	to 10 μ L total vol.
T ₄ DNA ligase	2.5 units

Set up the reactions on ice.

Incubate at 15°C for 16-20 h.
Add 1 μ L of 3M Na acetate and 27 μ L
absolute ethanol.
Store at -20°C until needed.

In vitro packaging of ligation mixtures:

Centrifuge above reaction mixture for 15 min.
To the dry pellet add about 2.5 μ L TE buffer.
Packaging reactions can be purchased from many different supplies. For our studies, we have tried the ones from Amersham as well as those from Stratagene. In our experience the "Gigapack Gold" packaging extracts (Stratagene) resulted in the highest pfu/mL. For the above λ gt10 vector system, 0.5 mL of SM buffer is added to the ligation mixture, together with 10 μ L chloroform. After mixing gently, the phage stock is stored at 4°C.

For λ ZAP vector

Reaction mix:

Linkered insert cDNA	100-500 ng (2.5 μ L or less)
λ ZAP/ <i>Eco</i> RI arms	1 μ g (1.0 μ L)
10X T ₄ DNA ligase	
buffer	0.5 μ L
T ₄ DNA ligase	3 units
H ₂ O	to 5 μ L final volume
Place reaction at RT for 1 h and at 4°C overnight.	

The λ ZAP vector is suitable for inserts ranging from 0 to 10 kb. As the λ ZAP arms are about 41 000 bp in length, 0.1 μ g of 4000 bp cDNA would be used for every 1 μ g of arms. In our studies we packaged 1 μ L of the above ligation mix using the Gigapack Gold packaging extracts from Stratagene. After packaging, 0.5 mL of SM buffer is added together with 20 μ L chloroform.

6.1.3 Titration of recombinants

For λ gt10 vector

To prepare plating cells:

1. Streak *E. coli* L87 and NM514 glycerol stocks on separate L-agar plates and incubate at 37°C overnight.
2. Pick an individual colony and inoculate into 10 mL L-broth + 0.4% maltose. Incubate with shaking overnight.
3. Add 1 mL to 50 mL L-broth + 0.4% maltose. Incubate at 37°C with shaking until an OD₆₀₀ of 0.5 is reached.
4. Centrifuge at 3000 rpm for 10 min. and resuspend in 15 mL ice-cold 10mM MgSO₄.
5. Mix and store at 4°C. Use within a few days.

For titration, dilutions are made of the final packaged phage mixture; eg., 30 μ L added to 270 μ L of SM buffer would be a 10² dilution. Duplicate dilutions are made for both types of plating cells. Some controls should be included, such as λ gt10 DNA and λ gt arms without insert. For the λ gt10 DNA control, the titre on L87 reflects the efficiency of the packaging reaction and should be greater than 2x10⁸ pfu/ μ g. The degree of biological selection is obtained from the ratio of the L87 (non-selective host) and NM514 (the selective host) titres and should be over 100.

For those controls using λ gt10 arms (no insert DNA), one obtains the figure for the arms selective ratio (ratio of L87:Nm514 titres; usually >100) and the background titre on NM514. Thus, for individual cDNA reactions, the L87 titre is divided by the arms selective ratio to obtain the true background on NM514 if no recombinants had been formed. The number of recombinants is obtained by subtracting the background value from the NM514 titre. The percentage recombinants on NM514 would represent the difference of the NM514 titres, before and after subtraction of the background value. The value for the number of recombinants per μ g cDNA can now be obtained. In our studies we have also used *E. coli* C600 strain with good results (eg. Pitel and Cheliak 1988b).

To check the size of the cDNA inserts, and to obtain another value for percentage recombinants, random plaques are picked out, the DNA extracted,

and analyzed by agarose electrophoresis as described below (sections 6.3 and 6.5).

For λ ZAP vector

To prepare plating cells:

1. Streak *E. coli* BB4 and XL-1 Blue cells on LB/tetracycline (12.5 $\mu\text{g}/\text{mL}$) plates and grow overnight at 37°C.
2. Pick an individual colony and grow overnight with shaking at 30°C in TB media together with 0.2% maltose and 10mM MgSO_4 .
3. Centrifuge cells at 1000 g for 10 min. and gently resuspend in 10mM MgSO_4 (0.5 volumes).

BB4 is a RecA+ strain. The XL1-Blue is a RecA-strain and is used for clones requiring a recombination minus strain. Both strains contain the F' episome, needed for both colour selection and automatic excision to a pBluescript plasmid following superinfection with an M13 helper phage.

For the final packaged mixture plate 1 μL of the final packaged reaction (in SM buffer) on a BB4 bacterial lawn:

1. Incubate phage and 200 μL of an $\text{OD}_{600} = 0.5$ BB4 cells for 15 min. at 37°C.
2. Add 2-3 mL top agar (48°C) and 50 μL of 0.5M IPTG plus 50 μL of 250 mg/mL X-Gal.
3. Plate onto NZY agar plates and leave at 37°C for 6-16 h. Background plaques are blue.

For the automated excision protocol of phagemid DNA:

1. Core plaque and transfer to 1.5 mL microcentrifuge tube containing 500 μL SM buffer and 20 μL chloroform. Vortex to release λ ZAP phage particles. Store at 4°C.
2. In a 50 mL tube add

200 μL of $\text{OD}_{600} = 1.0$ XL1-Blue cells
200 μL of ZAP phage stock
10 μL of R408 helper phage
Incubate at 37°C for 15 min.

3. Add 5 mL 2X YT media and incubate 4-6 h at 37°C with shaking.
4. Heat at 70°C for 20 min., then spin for 5 min. at 1000 rpm.
5. Decant the pBluescript plasmid (packaged in the M13 or f1 phage particle) into a sterile tube. Store at 4°C and use within 2 months.
6. To plate the rescued plasmid, add in a 15 mL tube:

200 μL $\text{OD}_{600} = 1.0$ of XL1-Blue cells
200 μL of phage stock from step 5

Incubate at 37°C for 15 min.

7. Streak on LB/Ampicillin plates and incubate overnight at 37°C. Colonies contain the p-Bluescript plasmid with the cloned insert.

Extraction of pBluescript plasmids

To obtain a double-stranded DNA template suitable for restriction enzyme digestion or enzyme sequencing:

1. Grow a 3 mL culture overnight in LB medium plus ampicillin (100 $\mu\text{g}/\text{mL}$).
2. Pellet 1.5 mL for 3 min. at 4°C.
3. Resuspend pellet in 110 μL STETL buffer

Sucrose	8%
Triton X-100	5%
Tris-HCl, pH 8.0	50mM
EDTA	50mM

Lysozyme is added as a powder at the end (~50 μg).

4. Place tube at 100°C for 30 sec.
5. Centrifuge at 4°C for 15 min.
6. To the supernatant, add 110 μL isopropanol and centrifuge immediately for 15 min.
7. Resuspend in 25 μL TE for restriction digestion or for further purification.

8. Extract with an equal volume of phenol:chloroform (1:1) twice, and once with chloroform alone.
9. Add an equal volume of 7.5M ammonium acetate and precipitate with 2.5 volumes of ethanol. Leave on ice and spin at 4°C for 20 min.
10. Rinse with 1 mL 80% ethanol and spin for 1 min.
11. Vacuum dry pellets and resuspend in 15 µL of 10mM Tris-HCl, pH 7.5; 0.1mM EDTA.
12. For sequencing use 5 µL (~2 µg).

Note: Other methods can be used, either on a small or large scale basis (eg., Section 6.5; "BIO 101" kit from Bio/Can; Maniatis et al. 1982).

Preparation of ssDNA from cells containing pBluescript plasmid

1. Grow an overnight culture containing p-Bluescript plasmid DNA (also use the p-Bluescript plasmid without insert as a control) in XL1-Blue. To select for the F'-episome grow the culture in 10 µg/mL tetracycline. Use the overnight culture immediately.
2. Inoculate 3 µL of 2X YT with 300 µL of the overnight culture and shake at 37°C in a sterile 50 mL tube.
3. Add helper phage (eg. R408 from Stratagene) to the culture during log phase ($OD_{600} = 0.3$) at a multiplicity of infection of 20:1 (phage to cells). Continue shaking for 8 h.
4. Transfer to an Eppendorf tube and spin for 15 min. Transfer the supernatant to a fresh tube and spin again for 15 min.

Transfer the supernatant to a fresh tube and add 1/4 volume of 3.5M NH_4Ac , pH 7.5; 20% PEG.

Invert to mix and leave at RT for 15 min.
5. Spin tubes for 20 min. at 11 000 rpm and drain supernatant. Spin briefly and remove any remaining supernatant.

6. Resuspend the pellet in 200 µL TE buffer. Add 200 µL buffer-saturated phenol/chloroform, vortex for 1 min., and spin for 5 min.
7. Remove top aqueous phase and transfer to a fresh tube. Repeat extractions until there is no interface.

Add 200 µL chloroform, vortex for 1 min., and spin for 2 min. Transfer the aqueous phase to a fresh tube.
8. Precipitate the ssDNA by adding 150 µL 7.5M NH_4Ac , pH 7.5 and 600 µL cold 100% ethanol. Leave on ice for 15 min.
9. Spin for 20 min at 4°C, drain supernatant, and wash pellet with 80% ethanol. Vacuum dry and dissolve pellet in 20 µL dH_2O . Transfer to fresh tube.
10. Analyze ssDNA on a 1% agarose gel. The VCS M13 and R408 run at ~4Kb while the pBluescript with no insert runs at ~1.6Kb.

6.2 Small scale extraction of phage DNA

6.2.1 Liquid culture method

1. With a sterile Pasteur pipette remove a plaque and add to 0.5 mL of fresh NM514 plating cells (as described in section 6.1.3) in a sterile 15 mL polypropylene tube. Leave for 15 min. at RT.
2. Add 5 mL of L-broth containing 5mM $CaCl_2$ (add 1 mL sterile 500mM $CaCl_2$ to 100 mL of cool sterile L-broth).
3. Incubate 4 h at 37°C with shaking.
4. Add a few drops of chloroform and shake for a further 5 min.
5. Centrifuge at 3000 rpm for 10 min. and add supernatant to 15 mL round bottom polypropylene tube (eg. Falcon). Store at 4°C.
6. To the above supernatant add an equal volume of 20% PEG, 2M NaCl in SM buffer. Mix and incubate 1 h on ice.

7. Centrifuge at 3000 rpm for 20 min. Drain pellet and wipe the inside of the tube to remove all traces of PEG.
8. Resuspend pellet in 750 μ L L-broth. Add to a 1.5 mL microcentrifuge tube.
9. Add 750 μ L of DE52 in L-broth. Gently mix by inversion 30 times.
10. Centrifuge for 5 min. and add supernatant to a new tube. Repeat this step.
11. To 750 μ L of supernatant add 13 μ L of a 0.1 mg/mL solution of proteinase K and 32 μ L of 10% SDS. Mix and leave at RT for 5 min.
12. Add 130 μ L of 3M potassium acetate. Invert several times and incubate at 88°C for 20 min. Cool on ice for 10 min.
13. Centrifuge for 10 min. and transfer 800 μ L of supernatant to a fresh tube. Add an equal volume of cold isopropanol and keep at -70°C for at least 10 min.
14. Centrifuge for 10 min., dry pellet briefly, and resuspend phage DNA in TE buffer. For *Hind*III digestions, only 5-10% of the pellet need be analyzed. For *Eco*RI digests and for non-radioactive analysis by electrophoresis the whole pellet is used.

6.2.2 Plate lysate method

1. Core out a plaque using a sterile Pasteur pipette and add to 1 mL of SM buffer.
2. Add a drop of chloroform, shake, and leave at 4°C for 1 h.
3. Adsorb about 10^5 pfu onto fresh suitable cells (eg. 514) and plate.
4. Incubate at 37°C for 8 h to overnight, add 5 mL SM buffer, seal, and shake gently at 4°C for 2-3 h.
5. Remove SM lysate into a sterile tube. Rinse the plate with 1 mL of SM buffer.
6. Add a few drops of chloroform, vortex briefly and spin for 10 min. at 4°C at 4000 rpm.

7. To the supernatant add one drop of chloroform and store at 4°C.

6.3 Amplification and storage of libraries

For studies using λ gt10 as vector:

1. Plate phage on NM514 or C600 cells at 3×10^5 recombinants per large square petri dish (eg. Nunc, 23x23 cm).
2. Incubate at 37°C until the plaques appear and merge. The plaques should not become too large.
3. Add 50 mL of SM buffer per plate and leave at 4°C overnight.
4. Remove buffer, wash plate with 10 mL of SM buffer, and store over chloroform at 4°C. Titres should be up to 5×10^{11} pfu/mL.

For studies with λ ZAP as vector:

1. Place phage on BB4 (or the *recA*- strain, XL1-Blue if the occurrence of recombination events are a concern) cells at 3×10^5 recombinants per large square plate. Aliquots of the packaged λ clones are mixed with 2 mL of $OD_{600} = 0.5$ /mL BB4 cells in a 50 mL polypropylene tube.
2. Incubate tubes for 15 min. at 37°C.
3. Mix 30 mL of melted (48°C) top agar with the infected bacteria and spread on NZY plates of bottom agar.
4. Incubate plates at 37°C for 5-8 h until the plaques appear and merge.
5. Add 40 mL SM buffer to each plate and keep at 4°C overnight.
6. Remove bacteriophage suspension and put into sterile polypropylene or glass containers. Rinse the plate with 10 mL fresh SM buffer and pool. Add chloroform to 5% and incubate at RT for 15 min.
7. Centrifuge at 4000 rpm for 5 min. to remove cell debris.

8. Recover the supernatant, pool, add chloroform to 0.3%, and store in aliquots at 4°C.

6.4 Characterization of cDNA inserts on agarose gels

For restriction digestion of phage DNA, digestion with *EcoRI* will release any insert. If these are difficult to see, the inserts can be radioactively labelled. Also, the DNA may be digested with *HindIII* or *BamHI*. For λ gt10 these enzymes release 6.5 Kb and 6.1 Kb fragments, respectively, which contain the *EcoRI* cloning site and any inserts. Any inserts present will alter the size of these bands. An example of this is presented in the reference by Pitel and Cheliak (1988b).

Digest reaction:

To 20 μ L of phage DNA add
 3 μ L enzyme buffer (x10)
 6 units enzyme
 X mL H₂O, to 30 μ L

Incubate at 37°C for 2h

Heat to 70°C for 10 min. and either load on a gel (1.5-2% agarose gel for *EcoRI* digests or 0.8% for *HindIII* or *BamHI* digests) or label as described below.

Labelling reaction:

0.1 μ L (α -³²P) dATP (10 μ Ci/ μ L)
 1 unit DNA polymerase I, Klenow fragment
 X μ L H₂O to 2.5 μ L
 Add the above to each 30 μ L *EcoRI* digest and incubate at 30°C for 30 min.
 Analyze on a 1.5% agarose gel
 Fix in 7% TCA for 30 min., dry, and autoradiograph

6.5 Mini-prep plasmid DNA preparation

The following method is essentially as described by Birnboim and Doly (1979).

1. With a sterile loop to transfer a single colony, inoculate 2 mL L-broth with an appropriate antibiotic and incubate overnight at 37°C with shaking.
2. Transfer 1 mL to a 1.5 mL microcentrifuge tube and centrifuge for 2-3 min. Resuspend pellet (eg. Pipetman) in 100 μ L of lysis solution:

25mM Tris-HCl (pH 8.0)
 10mM EDTA, (pH 8.0)
 50mM glucose
 2 mg/mL lysozyme (add just before use)
 Store on ice for 30 min.

3. Add 200 μ L of alkaline SDS at RT:

1% (w/v) SDS in 0.2M NaOH
 Mix by inverting the tube, and keep on ice for 5 min.

4. Add 150 μ L of 3M sodium acetate (pH 4.8 with glacial acetic acid) and mix gently by inverting the tube. Incubate on ice for 60 min.
 5. Centrifuge 5 min. at RT and transfer 400 μ L of supernatant (avoiding white ppt. that sometimes floats on top) to another tube.
 6. Add 1 mL of ethanol and store at -70°C for at least 1 h.
 7. Centrifuge at 4°C and carefully remove the supernatant.
 8. Resuspend pellet in 100 μ L of 0.1M ammonium acetate (pH 6.0), add 300 μ L of cold ethanol, and keep at -70°C for at least 30 min.
 9. Centrifuge and discard the supernatant as before. Dry the pellet and resuspend in TE buffer.
 10. For restriction enzyme digests RNase (20 μ g/mL) can be included.
- 6.6 Some notes on growth and storage of *E. coli* and λ

Growth of *E. coli* occurs as an initial lag phase, a period of exponential growth, and then a stationary phase. This growth or turbidity can be measured at 600 nm. Although *E. coli* can grow under anaerobic conditions, vigorous aeration is required to obtain the maximal growth rate in any given media. The culture volume should be less than 25% of the container size. Growth conditions should be kept constant to prevent proliferation of bacteria in different physiological states.

Storage of *E. coli* as colonies on L plates at 4°C should be for less than 10 days because there is rapid loss of cell viability. Strains that are used frequently

can be stored at 4°C in MgSO₄ (10mM) suspension for up to one month (Silhavy et al. 1984). However, in the protocol described by Amersham for NM514 cells, the suggestion is to use only freshly prepared plating cells, as storage of the cells at 4°C for up to one week leads to a significant loss of packaging efficiency and biological selection. For long-term storage it is best at -70°C, using screw-capped plastic freezing vials. To one vial add 0.4 mL overnight culture and 0.6 mL 20% glycerol. Make two or more vials for each sample and keep in separate freezers. Although vials can be frozen and thawed many times, it is best to scrape off a little frozen culture and streak it out.

In general, log-phase cells are required for lytic growth of λ , while stationary phase cells are used to make lysogens. To have overnight cells at log phase in the morning Silhavy et al. (1984) suggested incubation at 30°C to slow their growth, and use of a less rich medium, such as TB. In TB medium the cells stop growing at an OD₆₀₀ of about 0.5-0.6. They indicated also that L plates give smaller, less-uniform plaques than do TB plates. However, for liquid lysates of λ , L medium is needed. Bacteriophage λ adsorbs to an outer membrane protein LamB (the product of lamB gene) of *E. coli*. Strains that lack this protein are λ resistant (λ^r). The lamB gene is inducible by maltose. Thus, when cells are grown in 0.2% maltose, several thousand receptors result, compared with only a few hundred per cell when maltose is absent. Adsorption of λ is independent of temperature, while injection requires warm (eg. 37°C) temperatures. λ will not form plaques, therefore, at RT. Magnesium ions are also needed for stability and adsorption to *E. coli*, while Ca⁺⁺ is a competitive inhibitor. Adsorption can also occur with dead cells or debris if the LamB protein is present. Salt levels greater than 0.5M will inhibit adsorption.

The best method for storing λ is with plate or liquid culture stocks (see Section 6.3) that are kept in the dark and tightly sealed at 4°C. These can last for years. Although lysates are sterilized with chloroform, excess amounts should not be used because chloroform undergoes photolysis, yielding compounds lethal to λ . Lysates should be as free of bacterial debris as possible to reduce loss of titer through adsorption. Permanent storage is best achieved by freezing at -70°C or in liquid nitrogen. Use screw-capped, plastic freezing vials and add 70 μ L DMSO and 1 mL phage lysate. Freeze two or more vials per lysate, and store in two separate freezers if possible. Although λ can be frozen and thawed repeatedly with little or no loss of viability, it is best to open a frozen vial, scrape of a little frozen lysate with a sterile loop

or toothpick, and resuspend the sample in an appropriate buffer for plating.

7 Subtractive hybridization protocols

7.1 Preparation of labelled cDNA

The cDNA is prepared either to result in high specific activity for blot hybridization studies, or low specific activity for selected cDNA cloning (Davis et al. 1984).

Single strand cDNA reaction mixture (100 μ L):

1-5 μ g poly(A)⁺ RNA in 50mM Tris-HCl,
pH 8.3
6mM MgCl₂
60mM NaCl (or 70mM KCl for cDNA
cloning studies)
1mM dATP
1mM dGTP
1mM dTTP
500 μ Ci [α -³²P] dCTP (6mM final
concentration) for blot hybridization
studies, or only trace amounts for cDNA
cloning studies
30 mM dCTP (or 1mM dCTP for cDNA
cloning studies)
10 μ g/mL oligo dT
20mM dithiothreitol
100 μ g/mL actinomycin D
Add 10 units of reverse transcriptase per
 μ g poly(A)⁺ RNA and incubate for 2 h
at 42°C

Note: Other protocols for cDNA synthesis, for example that by Amersham, can also be used as described earlier.

1. To the above cDNA, add an equal volume of 0.2 M NaOH and incubate at 70°C for 20 min. to hydrolyze the RNA.
2. Cool on ice, neutralize with 1M HCl, add Na acetate (pH 6.5) to 0.2 M and NaDodSO₄ to 0.1%.
3. Warm to RT and exclude the labelled cDNA from Sephadex G-50F in a Pasteur pipette column with a running buffer of:

100mM NaCl
50mM Tris-HCl, pH 7.5
1mM EDTA
0.02% NaDodSO₄

4. Add 50 µg tRNA as carrier and precipitate the cDNA in a silanized Eppendorf tube.
5. Wash ppt with 70% ethanol, dry and resuspend in water.

Note: For blot hybridization studies the labelled cDNAs (1.8×10^8 CPM/µg) are left for two weeks to fragment the material.

Another method to prepare labelled cDNA for use only as a hybridization probe is described by Sargent (1987). Because high specific activity tracer cDNA ($>10^8$ cpm/µg) is needed, low concentrations of dNTPs are used in the reaction. Due to this requirement, priming is with random oligodeoxy-nucleotides rather than oligo (dT) to prevent artificial bias for 3'-proximal sequences owing to the smaller cDNAs being manufactured. Random primer cDNA is made as follows:

5 mCi/mL [^{32} P] dCTP (3000 Ci/mmol)
 20 µM unlabelled dCTP
 200 µM each of dATP, dGTP, and dTTP
 20mM DTT
 50mM Tris-HCl (pH 7.5)
 75mM KCl
 3mM MgCl₂
 10 µg/mL poly(A)⁺ RNA
 300 µg/mL random primer
 6000 U/mL MMLV reverse transcriptase (BRL)

Incubate for 1 h at 37°C and hydrolyze the RNA with an equal volume of 0.6N NaOH, 20mM EDTA and incubation at 65°C for 30 min., followed by chromatography in Sephadex G-50 in NETS buffer to remove unincorporated label. Using random primer conditions values of 10^{10} cpm of cDNA can be obtained from 0.5 µg poly(A)⁺ mRNA and should be subtracted with at least 10 µg of driver mRNA. Usually an RNA to cDNA ratio of 30:1 is used.

Only a few hundred thousand cpm of subtracted cDNA are needed to hybridize to libraries, as subtracted cDNA contains only sequences of interest and represents a small part of unfractionated cDNA. As background is reduced, long exposure times are possible. If over 95% of the original cDNA is removed one can detect rare mRNAs on the order of 0.01% or less of the total cellular mRNA. For an mRNA that is 0.01% of the total, 50 000 clones should be screened.

7.2 Construction of directionally cloned cDNA libraries and isolation of recombinant phage DNA

Following construction of cDNA using the oligo (dT) -XbaI primer - adaptor (Promega), addition of EcoRI linkers, and final digestion with both EcoRI and XbaI, the cDNAs are ligated to a suitable vector having compatible EcoRI-XbaI arms for direction cloning. In our studies we have so far used the λ GEM-4 vector from Promega. The ligation reaction includes:

0.5 µg λ GEM-4 arms
 0.15 µg cDNA (or pGEMTM-3 test insert)
 1.0 µL 5X ligase buffer
 1.0 µL T4 DNA ligase
 H₂O to 5.0 µL final volume

To check background, omit the test insert.

Incubate the ligation mixture for 3 h at RT and then package according to manufacturers' instructions. To the packaging mix add 0.5 mL SM buffer and 25 µL chloroform. Titer the phage on TB plates using LE392 plating cells. A single colony of LE392 is added to 50 mL of TB medium supplemented with 0.5 mL 1M MgSO₄ and 1 mL sterile 20% maltose and then shaken overnight at 30°C. After centrifugation the bacteria are stored at 4°C for a few days in 10mM MgSO₄. For plating, 100 µL of diluted phage is added to 100 µL LE392 plating cells and left at 37°C for 30 min., followed by addition of TB top agar (45°C).

After the library is constructed, orientation-specific RNA probes can be made from individual clones or the entire library. In the following protocol from Promega about 30-150 µg of RNA is synthesized. To isolate recombinant phage DNA:

Method 1

1. Grow a 100 mL culture of phage lysate, or use plate lysates.
2. Centrifuge at 8000 rpm for 10 min. to remove cell debris.
3. Add 0.9 mL LambdaSorb(Promega)/90 mL lysate, mix, and put on ice for 30 min.
4. Centrifuge at 3000 rpm for 10 min. and resuspend pellet in 10 mL SM phage buffer. Centrifuge at 12 000 rpm for 2.5 min. and repeat.

5. Resuspend pellets in 10 mL of 10mM Tris-HCl, pH 8.0, and 10mM EDTA. Heat to 70°C for 5 min. to release phage DNA.
6. Centrifuge for 2 min. and transfer supernatant to another tube.
7. Extract once with one volume of phenol:chloroform (1:1) saturated in TE buffer. Vortex for 1 min. and centrifuge at 12 000 rpm for 5 min.
8. Extract the aqueous phase with 1.0 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 min. and centrifuge. Repeat extraction with chloroform.
9. To the aqueous phase, add 0.5 vol. of 5M NaCl and mix.
10. Add 0.33 volumes of 30% PEG (MW 8000) in 1.5M NaCl. Mix well and put on ice for 30 min.
11. Centrifuge for 5 min. at 12 000 rpm, wash pellet with 1 mL 70% ethanol, and then briefly dry the pellet.
12. Resuspend the DNA in 100 μ L H₂O. Yields are about 30-60 μ g per 100 mL liquid lysate.

Method 2

The following is a method described by J. Byrne (pers. comm.) and is a modification from literature sources.

1. Prepare three large Nunc plates (23x23 cm) using desired medium and plating cells. Use 7.5×10^6 pfu per large plate. Leave at 37°C (after adding the top agar, plating cells, and the required pfu) for 5-6 h until the plates have a mottled-looking lawn on them. Do not grow too long.
2. Cool the plates at 4°C and overlay with 40 mL λ diluent plus several drops of chloroform (λ diluent is 10mM Tris-HCl, pH 7.5; 10mM MgCl₂; 0.1mM Na₂ EDTA). Leave at 4°C overnight.
3. Remove the overlay solution and sediment bacterial debris by centrifugation at 10 000 rpm at 4°C for 15 min.

4. Pellet the phage by centrifugation at 23 000 rpm at 4°C for 90 min.
5. Resuspend each pellet in 0.5 mL λ diluent overnight. Add CsCl to 0.75 g for each mL.
6. Centrifuge at 54 000 rpm at 4°C for 6 h.
7. Collect the band from the tube and dialyze overnight at 4°C with TE buffer (two changes).
8. Put 0.5 mL in each microfuge tube.
9. Add 10 μ L 10% SDS and 1 drop of Proteinase K (3 mg/mL, stored in glycerol at -20°C).
10. Incubate at 37°C for 30 min.
11. Extract with phenol:chloroform (2X) and chloroform (1X). Add 1/10 volume of 3M Na acetate and two volumes of cold ethanol.
12. Centrifuge and dissolve pellet in 500 mL TE. This may take 1-2 days at 4°C. Use 5 μ L for O.D. measurements.

Digestion of DNA:

1. Digest 30 μ g of DNA with *Xba*I (to transcribe with T7 RNA polymerase and thus generate sense RNA) for 1 h at 37°C.
2. Extract once with phenol:chloroform (1:1) and twice with chloroform: isoamyl alcohol (24:1).
3. Precipitate the DNA with 0.5 volumes of 7.5M NH₄ acetate followed by two volumes of ethanol.
4. Place at -20°C for 30 min. and centrifuge at 12 000 rpm for 15 min.
5. Wash pellet in 70% ethanol and dry under vacuum. Resuspend pellet in 30 μ L H₂O.

7.3 Transcription reaction

1. To 30 μ L of digested DNA, add:
 - 60 μ L 5X transcription buffer
 - 30 μ L 100mM DTT
 - 75 μ L 2.5mM each of ATP, GTP, UTP, CTP

12 μ L RNasin, 300 U
15 μ L T7 RNA polymerase, 300 U

2. Add DEPC-treated H_2O to a final volume of 300 μ L, and incubate at 37°C for 2h.
3. Add 30 units of RNase-free RQ1 DNase and incubate for 15 min. at 37°C.
4. Stop the reaction by adding an equal volume of phenol:chloroform (1:1) saturated with TE buffer.
5. Vortex for 1 min. and centrifuge at 12 000 rpm for 5 min.
6. Extract the aqueous phase with an equal volume of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge.
7. To the aqueous phase, add 0.5 volume of 7.5M NH_4 acetate followed by three volumes of ethanol.
8. Place at -20°C for 30 min. and centrifuge at 12 000 rpm for 15 min.
9. Wash pellet in 70% ethanol and dry under vacuum. Resuspend the pellet in 100 μ L of DEPC-treated H_2O and store at -20°C or -70°C.
10. Make cDNA using reverse transcriptase and later base hydrolysis.

7.4 Hybridization reactions

1. Silanize glass capillaries (to hold 20 μ L of solution), or use silanized screw-capped microfuge tubes topped off with paraffin oil and closed.
2. Dissolve mRNA (at 1 μ g/ μ L) in 0.5M phosphate buffer containing 5mM EDTA and 0.1% Na dodecyl sulphate.
3. Boil for 60 sec. to disaggregate the RNA.
4. Incubate reactions at 68°C for 20-24 h.
5. Fractionate by hydroxyapatite chromatography in 0.12M phosphate buffer containing 0.1% Na dodecyl sulphate at 60°C. The cDNA (double-stranded fraction) binds to the column.

6. Concentrate the single-stranded fraction with sec-butanol, extract with $CHCl_3$ to remove the butanol, and then aerate to remove the $CHCl_3$.

For blot hybridization studies, the material after step No. 7 is then hybridized to Southern blots.

7. Pass the single-stranded material through Sephadex G-50F (Pasteur pipette column) to remove the phosphate buffer which would inhibit precipitation.
8. Add 50 μ g tRNA carrier and precipitate the single-stranded cDNA.
9. Resuspend in H_2O and make double stranded, using standard procedures. Proceed with cloning. Further protocols can be obtained from Promega, such as for the requirement of T7 spacer primer

Invitrogen has available a cDNA subtraction kit which it claims is an improvement over other methods. This procedure does not require directional cDNA cloning. In this procedure two libraries are first constructed in phagemid vectors, one from tissue A (induced tissue) and the other from tissue B (uninduced tissue). ss DNA is then purified from both libraries using M13 helper phage. The ss DNA from library B is photobiotinylated, then hybridized with the ss DNA from library A. The resulting biotinylated duplex DNA and ss DNA from tissue B are removed by treatment with streptavidin followed by phenol extraction. The streptavidin-photobiotin-duplex DNA partitions into the phenol layer while the unhybridized ss DNA from tissue A remains in the aqueous layer and is then transformed into competent *E. coli* to produce a subtraction library.

Notes: Hydroxylapatite, a form of insoluble calcium phosphate, binds double-stranded molecules more tightly than single-stranded ones. Both ss and ds cDNA molecules can be released with phosphate buffers. Chromatography is carried out in a water-jacketed column kept at 55-60°C, containing 0.5 g hydroxylapatite (HTP grade, Bio-Rad) per 100 μ g RNA. The terminated hybridization reaction is diluted into 0.5 mL of 0.12M NaH_2PO_4 (pH 6.8) and applied to the column. Single-stranded cDNA will pass through, and residual single-stranded material is recovered by washing the column with several volumes of 0.12M NaH_2PO_4 .

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Appendix I

Stock solutions

Ammonium acetate, 4M

Add 308.3 g ammonium acetate to 800 mL H₂O. Adjust volume to 1 L. Sterilize by filtration and store at 4°C.

Ampicillin

The stock solution is 25 mg/mL of the sodium salt of ampicillin in H₂O. Sterilize by filtration and store in aliquots at -20°C.

For plates, cool autoclaved media to 48°C before adding ampicillin to a final concentration of 35-50 µg/mL. Store at 4°C for only 1-2 weeks before use.

Binding buffers for mRNA isolation

To 400 mL water add 0.61 g Trizma base
Adjust pH to 7.6 with 1N HCl.
Add 0.17 g EDTA and 0.50 g SDS
Dilute to 500 mL

- (a) 1X Binding Buffer (10mM Tris-HCl, pH 7.6; 1mM EDTA; 0.4M NaCl; 0.1% SDS).

To 200 mL of the above add 4.68 g NaCl and autoclave.

- (b) 2X Binding Buffer (10mM Tris-HCl, pH 7.6; 1mM EDTA; 0.8M NaCl; 0.1% SDS).

To 100 mL of the above add 4.68 g NaCl and autoclave.

- (c) Elution Buffer (10mM Tris-HCl, pH 7.6; 1mM EDTA; 0.1% SDS).

200 mL of the above, and autoclave.

Chloroform:isoamyl alcohol (24:1, v/v)

In a large screw-capped bottle, add 240 mL of chloroform and 10 mL isoamyl alcohol. Mix and wrap the bottle in aluminum to protect from light. Store, tightly capped, at RT.

DEAE-cellulose (Whatman) DE52

Add 100 g of DE52 to a beaker and slowly add several volumes of 0.05N HCl until the pH is below 4.5. While gently stirring, slowly add concentrated NaOH until the pH is about 7.5. Allow resin to settle, decant the supernatant, and wash the resin several times with L-broth (resuspend the resin with two volumes of L-broth, followed by settling and decanting, until the pH of the supernatant is 7.5). Resuspend to make a slurry of about 75% resin and 25% L-broth. Add sodium azide to 0.1% and store at 4°C.

Denaturing solution

NaCl	86.7 g
NaOH	20.0 g (add to H ₂ O with care)
H ₂ O	to 1 L

Denhardt's, 50X

Ficoll 400	1 g
polyvinylpyrrolidone	1 g
BSA	1 g
H ₂ O	to 100 mL

EDTA, 0.25M

Add 93.1 g of Na₂ EDTA.2H₂O to 800 mL H₂O. Stir and adjust to pH 8 with NaOH. Adjust volume to 1 litre. Dispense into aliquots and autoclave.

Ethidium bromide

25 mg/50 mL stock (store at 4°C in the dark)
 Use 1 in 1000 in gel buffer mix before pouring
 (wear gloves and handle with care as this is a mutagen)
 This chemical can also be purchasing as a solution (eg. Sigma) or as pellets

Formamide, deionized:

Add 50 mL formamide to 5 g of mixed-bed resin AG 501-XB, 20-50 mesh, fully regenerated (Bio-Rad Laboratories). After stirring gently for 1 h at 4°C, filter twice through Whatman No. 1 filter paper. The formamide is then sterilized by filtration, and small aliquots stored at -20°C. This method removes both salt impurities and hydrolysis products.

Gel loading buffer for DNA electrophoresis:

(1)

0.25% bromphenol blue
 0.25% xylene cyanol
 40% (w/v) sucrose in H₂O or 30% glycerol in H₂O
 Store in small aliquots at 4°C

(2)

0.25% bromphenol blue
 40% (w/v) sucrose in H₂O
 Store in small aliquots at 4°C

HCl, 1N

If the conc. reagent is 12N, 82.5 mL are needed to prepare a 1 L, 1N solution. Note: add acid to water.

IPTG (isopropylthio- β -D-galactoside)

Add 25 mg/500 μ L sterile dH₂O and store at -20°C. Use 10 μ L per 9 cm petri dish

KCl, 2M

To 80 mL H₂O, add 14.9 g KCl. Dilute to 100 mL. Add 50 μ L DEPC and autoclave.

L medium (LB, Luria-Bertani)

per litre:

Bactotryptone	10 g
Bactoyeast	5 g
NaCl	10 g

Dissolve in 800 mL H₂O. Adjust to pH 7 and make to 1L. Pour into 500 mL large screw-cap bottles. Autoclave.

L-agar plates

per litre:

Bactotryptone	10 g
Bactoyeast	5 g
NaCl	10 g
Bacto-agar	15 g

Dissolve in 800 ml H₂O. Adjust pH to 7 and volume to 1 L. Autoclave, mix, and cool to 50°C before pouring into the plates (~25 mL). Leave to set for ~1 h, invert, and store at 4°C or RT in plastic bags. Dry each plate briefly before use by placing at 37°C inverted with the lid removed.

L top agar

per litre:

Bactotryptone	10 g
Bactoyeast	5 g
NaCl	5 g
MgSO ₄	2.5 g
Bacto-agar	10 g

Dissolve in 800 mL H₂O, adjust pH to 7 and volume to 1 L. Autoclave, mix, and store at RT or at 50°C. Use only when cooled to 45-50°C. Agarose is used instead of Bacto-agar for plaque lift studies.

LiCl, 2M

To 80 mL H₂O add 8.48 g LiCl. Dilute to 100 mL. Add 50 μ L DEPC and autoclave.

LiCl, 10M

To 40 mL H₂O add 21.2 g LiCl. Dilute to 50 mL. Add 25 μ L DEPC and autoclave.

Maltose

maltose	20 g
H ₂ O	to 100 mL

Sterilize by filtration. Add 1 mL per 100 mL of medium.

NETS buffer

NaCl	0.1M
Tris-HCl, pH 7.6	10mM
EDTA	0.1mM
SDS	0.1%

Neutralizing solution

NaCl	87.7 g
1M Tris-HCl pH 7	500 mL

Dissolve first in 800 mL H₂O and then adjust volume to 1 L.

NZY broth (per litre)

NaCl	5 g
MgSO ₄ •H ₂ O	2 g
Yeast extract	5 g
NZ Amine (casein hydrolysate)	10 g

Autoclave

NZY plates

To 1 litre of NZY broth, add 15 g Difco Agar
Autoclave, and pour

NZY top agar

To NZY broth, add 0.7% agarose
Autoclave

Phenol

Use high quality phenol. For phenol saturated with TE, mix TE buffer and phenol to give a 1/1 mixture. Remove upper aqueous layer and add 8-hydroxyquinoline to the phenol to a concentration of 0.1% as an antioxidant. This phenol is used for studies with cDNA.

For studies involving RNA extraction, the phenol is melted after storage at -20°C and 8-hydroxyquinoline added (0.1% w/v). The phenol is then extracted several times with an equal volume of 1.0M Tris, pH 8.0, followed by 0.1M Tris, pH 8.0 and 0.2% mercaptoethanol, until the pH of the aqueous phase is 7.6. The phenol can be stored at 4°C for up to one month.

Potassium acetate, 3M

To 15 mL H₂O add 7.36 g K acetate. Adjust pH to 5.5 with acetic acid. Dilute to 25 mL, add 12.5 µL DEPC, and autoclave.

SDS, 10%

For a 10% (w/v) solution heat to 68°C to aid dissolution and adjust pH to 7.2 with HCl. Dispense into aliquots.

Sephadex G50

Slowly add 30 g of Sephadex G50 (medium) to 250 mL of TE buffer (pH 8.0) containing 0.1M NaCl in a 500 mL beaker or bottle. Make sure that the powder is well dispersed. Stand overnight at RT or heat at 65°C for 1-2 h, or autoclave for 15 min. Allow to cool to RT. Decant the supernatant and replace with an equal vol. of buffer. Store at 4°C in a screw-capped bottle.

SM buffer (per litre)

NaCl	5.8 g
MgSO ₄ · 7H ₂ O	2.0 g
1M Tris-HCl, pH 7.5	50 mL
2% gelatin	5 mL
Dissolve in 800 mL H ₂ O. Adjust volume to 1 L. Autoclave.	

Sodium acetate, 3M:

Dissolve 408 g of sodium acetate, 3H₂O in 800 mL H₂O. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 1 litre. Dispense in aliquots and sterilize by autoclaving.

SSC 20X conc.

NaCl	175.3 g
Tri-sodium citrate	88.2 g
Dissolve in 800 mL H ₂ O, adjust pH to 7 with NaOH and then adjust volume to 1 L.	

STE buffer

100mM NaCl	5.84 g/L
10mM Tris	1.21 g/L
1mM EDTA. 2H ₂ O	0.372 g/L
Adjust pH to 7.5 with HCl and autoclave	

T4 DNA ligase buffer (5X)

250mM Tris, pH 7.8
50mM MgCl ₂
100mM DTT
5mM ATP
250 µg/mL BSA

TAE buffer, 40X conc.

Tris	193.6 g
Na acetate $\cdot 3H_2O$	108.9 g
EDTA, $Na_2 \cdot 2H_2O$	15.2 g
Adjust pH to 7.2 with acetic acid and final volume to 1 L with H_2O .	

TB medium (tryptone broth, λ medium)

The glucose present in the yeast extract of LB medium inhibits synthesis of the λ phage receptor protein located on the surface of the cell. Thus, some λ mutants require the use of TB plates.

	per litre
Bactotryptone (Difco)	10 g
NaCl	5 g
Agar (Difco)	11 g if poured in plates 8 g for top agar
After autoclaving, add 10 mL of 1M $MgSO_4$	

TBE buffer (10X stock)

108 g Tris base
55 g boric acid
9.3 g $Na_2EDTA \cdot 2H_2O$
This gives a stock solution of pH 8.3. Make to 1 L with dH_2O .

TE buffer

Tris	1.21 g
EDTA $\cdot 2H_2O$	0.37 g
H_2O	to 1l
Adjust to pH 7.5 with HCl and autoclave	

Tetracycline

The stock solution is 12.5 mg/mL tetracycline hydrochloride in ethanol/water (50% v/v). Sterilize by filtration and store at $-20^\circ C$ in the dark.

For plates, cool autoclaved LB media to $48^\circ C$ before adding tetracycline to a final concentration of 12.5-15.0 $\mu g/mL$. Store plates in the dark at $4^\circ C$.

Transcription buffer (5X conc.)

Tris-HCl	200mM
$MgCl_2$	30mM
spermidine	10mM
NaCl	50mM
This is titrated to pH 7.9 at RT such that after dilution the pH will be 7.5 at $37^\circ C$.	

Tris, 1M at pH 8.0

Dissolve 121.1 g of Tris base in 800 mL H₂O. Adjust to pH 8 with HCl (about 42 mL). Make volume to 1 L.

Some drawbacks in using this buffer include: chelation of some ions; high temperature coefficient, so it is necessary to check the pH change upon cooling or heating a Tris buffer from room temperature; and, pH varies considerably with concentration.

Water, distilled

House-distilled water should be passed through an ion-exchanger first to remove impurities such as the aromatic amines which may remain. Glass lines, rather than tygon tubing, should be used to carry distilled water, as the latter can leach compounds into the water. Teflon stopcocks should be used rather than greased stopcocks. For critical experiments, HPLC Grade water can be purchased (eg. from Fisher Scientific).

X gal (5-bromo-4 chloro-3-indolyl- β -D-galactoside)

Dissolve X gal at 50 mg/500 μ L in N,N-dimethyl formamide in a glass vial. Store at -20°C in the dark. Use 25 μ L per 9 cm petri dish. For studies using the λ ZAP system, higher concentrations are needed.

YT broth

NaCl	5 g
Yeast extract	5 g
Bactotryptone	8 g
H ₂ O, to 1L	

Appendix II

Selected suppliers of chemicals, cells, and vectors

<i>Address</i>	<i>Types of supplies</i>
Aldrich Chemical Co. P.O. Box 212, Station A Windsor, Ontario N9A 6K1 (800) 558-9160 USA Aldrich Chemical Co., Inc. 940 West Saint Paul Avenue Milwaukee, WI 53233 (800) 558-9160/(414) 273-3850	General and rare chemicals
American Type Culture Collection (ATCC) 12301 Parklawn Drive Rockville, MD 20852 U.S.A.	Isolates, collects, preserves and distributes cultures of living microorganisms and human and animal cells
Amersham Canada Limited 505 Iroquois Shore Road Oakville, Ontario L6H 2R3 (416) 842-2720/(800) 268-5061 (outside Ont., Que.) (800) 387-4060 (Ont., Que.) USA Amersham Corporation 2636 South Clearbrook Drive Arlington Heights, IL 60005 (312) 364-7100/(800) 323-9750	Cloning systems (cDNA, M13), translation systems, enzymes, radiolabelled chemicals, "Hybond mAP" for poly(A) ⁺ isolation
AMRESCO (American Research Products Company) P.O. Box 39098 30175 Solon Industrial Parkway Solon, OH 44139 (800) 448-4442	Chemicals for biotechnology
Anachemia Canada, Inc. C.P./P.O. 147 Lachine Montreal, Québec H8S 4A7 (514) 489-5711/(800) 361-0209 (416) 279-5122 (Toronto) USA Anachemia Chemicals Inc. P.O. Box 783 Champlain, NY 12919 (513) 298-4444/(800) 323-1414	General and biotechnology reagents

<i>Address</i>	<i>Types of supplies</i>
BDH Chemicals 350 Evans Avenue Toronto, Ontario M8Z 1K5 (416) 255-8521/(800) 268-2129	General chemicals, "Difco" culture media products, eg., tryptone, yeast extract
Beckman Inc. 2500 Harbor Boulevard Fullerton, CA 92634 U.S.A.	Enzymes and reagents, antibodies
Bio/Can Scientific Inc. (Promega) 2170 Dunwin Drive, Unit 5 Mississauga, Ontario L5L 1C7 Canada (416) 828-2455/(800) 387-8125 USA Promega 2800 S. Fish Hatchery Road Madison, WI 53711 (608) 274-4330/(800) 356-9526	Transcription, sequencing, cloning, translation, and electroporation systems; enzymes; "Ultra-pure" chemicals for biotechnology. Also suppliers of "B10 101" products (DNA purification systems)
Bio-Rad Laboratories (Canada) Ltd. 3140 Universal Drive Mississauga, Ontario L4X 2C8 (416) 624-0713 USA Bio-Rad Laboratories, Inc. 1414 Harbour Way South Richmond, CA 94804 (800) 227-5589/(800) 227-3259 (inside CA)	Ion-exchange resins, gel filtration media, enzymes, cloning vectors, immunology products
Boehringer Mannheim Canada Ltd. 11450 Côte de Liesse Dorval, Québec H9P 1A9 (514) 636-6760/(800) 361-2070 (East) (800) 361-6046 (West) USA Boehringer Mannheim Biochemicals 7941 Castleway Drive Indianapolis, IN 46250 U.S.A.	Enzymes, cloning vectors, RE linkers, assorted reagents. nonradiative DNA labelling and detection kit
BRL (see GIBCO/BRL)	

<i>Address</i>	<i>Types of supplies</i>
Calbiochem Corporation P.O. Box 12087 San Diego, CA 92112-4180 (619) 450-9600 (800) 854-3417 U.S.A. (In Canada distributed by Terochem)	Miracloth, enzymes, chemical reagents
Caledon Laboratories Ltd. 40 Armstrong Avenue Georgetown, Ontario L7G 4R9 (800) 268-7451/(416) 877-0101	General chemicals, distributors of GFS chemicals
Canadawide Scientific Ltd. 1230 Old Innes Road, Unit 414 Ottawa, Ontario K1B 3V3 (613) 741-6197/(800) 267-3787	General chemicals, "Difco" culture media products, Eastman Kodak products J.T. Baker and Nalge Co., Inc., products
Canlab AHS Canada Inc. 2390 Argentina Rd. Mississauga, Ontario L5N 3P1 (416) 821-9660	General chemicals, eg., Mallinckrodt, J.T. Baker products; Difco laboratory products
Chemical Dynamics Corporation P.O. Box 395 3001 Hadley Road South Plainfield, NJ 07080 U.S.A. (201) 753-5000	General chemicals, resins
Clontech; Western Canada dist., Sin-Can Inc. 4015 1st Street, SE, #3 Calgary, Alberta T2G 4X7 (403) 287-2345 USA Clontech Laboratories, Inc. 4030 Fabian Way Palo Alto, CA 94303 (800) 662-CLON (outside CA) (415) 424-8188	Libraries, poly(A)⁺ RNAs, cloning and expression vectors, GUS gene fusion systems, reagents
Collaborative Research 128 Spring Street Lexington, MA 02173 U.S.A.	Peptides and nucleotide-related products

<i>Address</i>	<i>Types of supplies</i>
Consolidated Alcohols P.O. Box 372, Terminal A Toronto, Ontario M5W 1C8	Absolute ethyl alcohol
Cooper Biomedical 1 Technology Court Malvern PA 19355 U.S.A. <i>Canada</i> ICN Canada Ltd. 1956 Bourdon St. Montreal, Québec H4M 1V1 (514) 744-6792 (Montreal) (416) 848-2700 (Toronto)	Enzymes and antibodies
Diagnostic Chemicals Limited West Royalty Industrial Park Charlottetown, PEI C1E 1B0 (902) 566-1396	Enzymes, speciality chemicals, eg., X-Gal
Difco Laboratories P.O. Box 1058 A Detroit, MI 48232 U.S.A.	Bacterial culture media chemicals
DuPont Canada Inc., (NEN Products) 7070 Mississauga Road Box 2200-Streetsville Mississauga, Ontario L5M 2H3 (416) 821-5001 <i>USA</i> DuPont Company Biotechnology Systems P.O. Box 80024 Wilmington, DE 19880-0024 U.S.A. (800) 551-2121	"Gene Screen" membranes, cDNA synthesis kit, "Nensorb 20" (nucleic acid purification cartridge), cloning vectors, radiolabelled reagents, translation and sequencing systems, immunology products
Eastman Kodak Company 343 State Street Rochester, NY 14650 U.S.A. (716) 458-3702 <i>Canada</i> Products available from Fisher Scientific Co., Limited and Sargent-Welch of Canada, Ltd.	General chemicals, electrophoresis products, guanidine thiocyanate, dyes, stains

<i>Address</i>	<i>Types of supplies</i>
ESBE Laboratory Supplies 80 McPherson St. Markham, Ontario L3R 3V6 (416) 498-0575	Distributors of products from companies such as Ames, Costar
Fisher Scientific 112 Colonnade Road Nepean, Ontario K2E 7L6 (613) 225-6752 USA Fisher Scientific 711 Forbes Avenue Pittsburgh, PA 15219 (412) 562-8543	General chemicals, "Fisher Biotech" chemicals
Fluka Chemical Corp. 980 South Second Street Ronkonkoma, NY 11779 U.S.A. (516) 467-0980/(800) 358-5287 (800) 458-5287 Canada Products distributed by Caledon Laboratories, Terochem	General chemicals
FMC Bio Products 5 Maple Street Rockland, ME 04841-2994 U.S.A. (800) 341-1574 Canada Products available from Mandel Scientific Ltd.	Special and "purified" agaroses, ampholytes, acrylamide gel products
Gentec Inc. 2625 rue Dalton Ste. Foy, Québec G1P 3S9 (418) 657-8000	
GIBCO/BRL Canada Life Technologies, Inc. 2270 Industrial Street Burlington, Ontario L7P 1A1 (416) 335-2255/(800) 2634-6231	Cloning vectors, cDNA synthesis kits, enzymes, reagents, translation and sequencing systems, immunology products, nonradioactive nucleic acid detection system, oligonucleotides (eg., molecular linkers), frozen competent cells, "Gibco" products (culture media)

Address**Types of supplies****USA**

Bethesda Research Laboratories
Life Technologies, Inc.
P.O. Box 6009
Gaithersburg, Maryland 20877
(800) 638-8992/(301) 840-8000

ICN Biomedicals, Inc.
3300 Hyland Avenue
Costa Mesa, CA 92626
U.S.A.
(714) 545-0113

Canada

ICN Canada Ltd.
1956 Bourdon St.
Montreal, Quebec
H4M 1V1
(514) 744-6792

International Biotechnologies (Canada)
Inc. (IBI)
366 Adelaide St., E., Suite 536
Toronto, Ontario
M5A 3X9
(800) 387-1525/(416) 362-8278
(Products are now supplied through Terochem)

USA

International Biotechnologies, Inc. (IBI)
P.O. Box 9558
275 Winchester Avenue
New Haven, CT 06535
(203) 786-5600/(800) 243-2555

Invitrogen
11588 Sorrento Valley Rd., Suite 20
San Diego, CA 92121-9776
U.S.A.
1-619-259-4868
FAX: 1-619-259-8683

Johns Scientific Inc.
175 Hanson Street
Toronto, Ontario
M4C 1A7
(416) 699-5555/(800) 268-4410

LKB Instruments
9319 Gaither Road
Gaithersburg, MD 20877
U.S.A.
(301) 963-3200

Radiochemicals, electrophoresis
reagents, "ultra-pure" chemicals,
nylon transfer membrane (Biotrans)

Enzymes, sequencing reagents, linkers,
cloning vectors, custom oligonucleotide
synthesis, custom sequencing and
cloning

cDNA library cloning system,
subtractive hybridization kit, mRNA
isolation kit

Distributors of J.T. Baker reagents and
laboratory supplies

Electrophoresis reagents

Address**Types of supplies****Canada**

Products available from
Pharmacia (Canada) Inc.

Mandel Scientific Company Ltd.
Hanlon Business Park
2 Admiral Place, R.R. #6
Guelph, Ontario
N1H 6J3
(519) 763-2145

Assorted chemicals, eg., DEAE-cellulose, FMC products (eg., special agaroses and acrylamide)

New England Biolabs (NEBL)
32 Tozer Road
Beverly, MA 01915-5510
U.S.A.
(508) 927-5054

Cloning vectors, DNA enzymes, primers, linkers, adaptors

Oxford Laboratories of Canada Ltd.
289 Blakie Road
London, Ontario
N6L 1B8
(519) 652-2400

Supplies chemicals from companies such as J.T. Baker, Eastman Kodak

Pharmacia (Canada) Inc.
500 Morgan Boulevard
Baie D'Urfé, Québec
H9X 3V1
(514) 457-6661 / (800) 363-7878 (Québec, Ontario, Maritimes)
(800) 363-6877 (Nfld., Western Canada)
USA
Pharmacia Inc.
800 Centennial Avenue
Piscataway, NJ 08854
(201) 457-8000

Agaroses, carrier ampholytes, cell biology products, cloning vectors, cDNA synthesis kit, immunology products, oligodeoxynucleotides

Promega (see Bio/Can Scientific Inc.)

Schwarz Mann
56 Rogers Street
Cambridge, MA 02142
U.S.A.

General chemicals

Canada

Products available from
Canadian Scientific Products Ltd.
P.O. Box 7177
London, Ontario
N5Y 4J9
(519) 473-2277

<i>Address</i>	<i>Types of supplies</i>
Seikagaku America, Inc. 9049 Fourth Street North P.O. Box 21517 St. Petersburg, FL 33724 U.S.A.	Enzymes
Sigma Chemical Company P.O. Box 14508 St. Louis, MO 63178 U.S.A.	General chemicals
Stratagene 11099 North Torrey Pines Road La Jolla, CA 92037 (800) 424-5444 (orders) (800) 548-1113 (technical support)	Cloning vectors, custom gene libraries, enzymes, gene characterization kits, accessories for radioactive work, "Stratalinker™ UV crosslinker"
Terochem (East) 2740-A Slough St. Mississauga, Ontario L4T 1G3 (800) 268-4944/(416) 673-0705 Terochem (West) 5729-92 Street Edmonton, Alta. T6E 3A4 (800) 661-6431/(403) 438-2222	General chemicals; suppliers of biotechnology products from ABN, Cruachem, Calbiochem, Serva and IBI
Toronto Research Chemicals 4483 Chesswood Drive Downsview, Ontario M3J 9Z9	General chemicals; eg., cesium chloride
United States Biochemical Corporation (USB) P.O. Box 22400 Cleveland, OH 44122 U.S.A. (800) 321-9322	Enzymes and reagents, DNA sequencing products
Vector Cloning Systems 3770 Tansy Street San Diego, CA 92121 U.S.A.	Cloning vectors, packaging extracts, and bacterial strains
Western Scientific Services Ltd. 11620 Horseshoe Way Richmond, B.C. V7A 4V5 (604) 274-4111	Suppliers of products from Difco Laboratories, Worthington Biochemicals, et al.

Appendix III

Suppliers of equipment and materials

<i>Address</i>	<i>Types of supplies</i>
Aldrich Chemical Co. P.O. Box 212, Station A Windsor, Ontario N9A 6K1 (800) 558-9160 USA Aldrich Chemical Company Inc. 940 West Saint Paul Avenue Milwaukee, WI 53233 U.S.A. (800) 558-9160/(414) 273-3850	Assorted equipment, such as glassware, heaters, safety equipment, tools, kits
Amicon Canada Ltd. 1226 White Oaks Boulevard Oakville, Ontario L6H 2B9 (416) 842-2520/(800) 387-4042	Membrane filtration, microseparation, ultrafiltration systems, chromatography, columns
BDH Inc. 350 Evans Avenue Toronto, Ontario M8Z 1K5 (416) 255-8521	Distributors of Kimble or Nagle products
Beckman Instruments (Canada) Inc. 1045 Tristar Drive Mississauga, Ontario L5T 1W5 (416) 670-1234/(800) 387-6799 USA Beckman Instruments, Inc. Scientific Instruments Division Campus Drive at Jamboree Boulevard Irvine, CA 92713 U.S.A.	Spectrophotometers, centrifuges, liquid scintillation products, pH meters, assorted chemicals
Becton-Dickinson Canada 2464 South Sheridan Way Mississauga, Ontario L5J 2M8 (416) 822-4820	Syringes
Biomed Instruments, Inc. 1020 South Raymond Avenue, Suite B Fullerton, CA 92631 U.S.A. (714) 870-0290	Soft laser scanning densitometers

Address**Types of supplies**

Bio-Rad Laboratories (Canada) Ltd.
 3140 Universal Drive
 Mississauga, Ontario
 L4X 2C8
 (416) 624-0713/(800) 268-0213
 USA
 Bio-Rad Laboratories, Inc.
 1414 Harbour Way South
 Richmond, CA 94804
 U.S.A.
 (415) 232-7000/(800) 227-5589

Assorted equipment and supplies, eg., electrophoresis, DNA sequence analysis, disposable "Poly-Prep" chromatography columns, "Zeta-Probe" blotting membranes, immunology products

Brinkmann Instruments (Canada) Ltd.
 50 Galaxy Blvd.
 Rexdale, Ontario
 M9W 4Y5
 (416) 675-7911
 USA
 Brinkmann Instruments Co.
 Division of Sybron Corporation
 Cantiague Road
 Westbury, NY 11590
 (800) 645-3050

"Polytron" homogenizer, temperature circulators and baths ("Lauda"), Eppendorf pipettes

BRL (see GIBCO/BRL)

Canadawide Scientific Ltd.
 1230 Old Innes Road, Unit 414
 Ottawa, Ontario
 K1B 3V3
 (613) 741-6197/(800) 267-3787

Assorted equipment, distributors of Thermolyne products and Nasco sampling equipment (eg. Whirl-Pak sampling bags)

Canlab AHS Canada Inc.
 2390 Argentina Road
 Mississauga, Ontario
 L5N 3P1
 (416) 821-9660

Assorted equipment and supplies (eg., products from Nalge Co. Inc., Whatman, Sartorius, Eberbach Corp.

Cole-Parmer Instrument Co.
 7425 North Oak Park Avenue
 Chicago, IL 60648
 U.S.A.
 (800) 323-4340 for both Canada and USA
 (312) 647-7600

Assorted equipment

Comeau Technique Ltd.
 5605 Vanden Abeele
 St-Laurent, Québec
 H4S 1S1
 (514) 334-3165/(800) 361-2553 (East)
 (800) 363-2841 (West)

Distributors for Thomas Scientific, Cole-Parmer, Hanna Instruments

<i>Address</i>	<i>Types of supplies</i>
Costar 205 Broadway Cambridge, MA 02139 U.S.A (800) 492-1110 <i>Canada</i> Products available from John's Scientific Inc.	Plasticware, eg., vials, polypropylene centrifuge tubes
Diamed Lab Suplies Inc. 3069 Universal Dr. Mississauga, Ontario L4X 2E2 (613) 233-1089/(416) 625-6021 (800) 387-7519	Assorted equipment, eg., power supplies, centrifuges, shakers, microcentrifuge tubes
DuPont Canada Inc. 7070 Mississauga Rd., Box 2200-Streetsville Mississauga, Ont. L5M 2H3 (416) 821-5001 <i>USA</i> DuPont Company Biotechnology Systems P.O. Box 80024 Wilmington, DE 19800-0024 (800) 551-2121	"Sorval" centrifuges, DNA and peptide synthesizers
Ealing Scientific Ltd. 6010 Vanden Abeele Street St. Laurent, Quebec H4S 1R9 (514) 335-0792/(800) 361-1905	Distributors of Harvard products; eg., clamps, lattices, pumps, autoclaves
Eastman Kodak Company Laboratory and Research Products Division 343 State Street Rochester, NY 14650 U.S.A. (716) 458-3702 <i>Canada</i> Products available from Fisher Scientific Co., Limited, and Sargent-Welch Scientific of Canada, Ltd. Also: Kodak Canada Gov't Sales 55 Metcalfe Street, Ste. 270 Ottawa, Ontario K1P 6L5	X-ray film and cassettes

<i>Address</i>	<i>Types of supplies</i>
Eberline Instrument Corp. P.O. Box 2108, Airport Road Santa Fe, NM U.S.A. (505) 471-3232 <i>Canada</i> Safety Supply Canada 214 King Street, East Toronto, Ontario M5A 1J8 (416) 364-3234	Radiation detection systems
E-C Apparatus Corporation 3831 Tyrone Boulevard, North St. Petersburg, FL 33709 U.S.A. (813) 344-1644/(800) 624-2232 <i>Canada</i> Products available from Mandel Scientific Company Ltd.	Electrophoresis products, densitometers
Elkay Products Inc. 800 Boston Turnpike Shrewsbury, MA 01545 U.S.A. (800) LAB-PROD/(508) 845-2116 <i>Canada</i> Products supplied by eg., Canlab, Ingram and Bell Scientific	Plasticware, eg., disposable transfer pipettes, centrifuge tubes, pipette tips
Falcon Labware Becton Dickenson Labware 1950 Williams Drive Oxnard, CA 93030 U.S.A. <i>Canada</i> Products available from eg., John's Scientific Inc.	Cell culture plasticware, eg., 16 mL sterile polypropylene tubes
Fisher Scientific 112 Colonnade Road Nepean, Ontario K2E 7L6 (613) 225-6752/(800) 267-1025 <i>USA</i> Fisher Scientific 711 Forbes Avenue Pittsburgh, PA 15219 (412) 562-8300	Assorted equipment: suppliers of "Lab-line" shakers, "IEC" centrifuges, Corning products, Nalgene labware, etc.

<i>Address</i>	<i>Types of supplies</i>
FTS Systems, Inc. P.O. Box 158 Stone Ridge, NY 12484-0158 (800) 251-1531/(914) 687-0071	Freeze-dryers
Gelman Sciences Inc. 2906 Diab Street Montreal, Québec H4S 1M7 (514) 337-2744/(800) 361-7868 (eastern Canada) (800) 361-6660 (western Canada) USA Gelman Sciences 600 Wagner Rd. Ann Arbor, MI 48106 U.S.A.	Filtration systems
GIBCO/BRL Canada Life Technologies Inc. 2270 Industrial Street Burlington, Ontario L7P 1A1 (416) 335-2255 USA Bethesda Research Laboratories Life Technologies, Inc. P.O. Box 6009 Gaithersburg, MD 20877	Electrophoresis (eg. sequencing) systems, nucleic acid blotting systems, distributors of Belco Glass products
Hoefer Scientific Instruments 654 Minnesota Street, Box 77387 San Francisco, CA 94107 U.S.A. (800) 227-4750/(415) 282-2307 (in CA) Canada Technical Marketing Associates 179 Colonnade Road, Suite 100 Nepean, Ontario K2E 7J4 (613) 226-8297 (Ottawa)/(416) 826-7752 (Toronto)	Electrophoresis systems, gradient makers, densitometers etc.
Horizon Environmental 7435 North Oak Park Avenue Chicago, IL 60648 U.S.A. (312) 647-7644 Canada Comeau Technique Ltd. 5605 Vanden Abeele St-Laurent, Québec H4S 1S1 (514) 334-3165/(800) 361-2553	Assorted equipment

Address**Types of supplies**

Ingram and Bell Scientific
20 Bond Avenue
Don Mills, Ontario
M3B 1L9
(416) 443-9647

Supplies products from Hitachi, Elkay,
Nucleopore, Gen-Probe, Puffer Hubbard,
ISS (electrophoresis supplies)

Integrated Separation Systems
One Westinghouse Plaza
Neponset Valley Parkway
Hyde Park, MA 02136
U.S.A.
(617) 361-5700

Electrophoresis supplies

International Biotechnologies Canada, Inc. (IBI)
366 Adelaide St. E. Suite 536
Toronto, Ontario
M5A 3X9
(800) 387-1525/(416) 362-8278
USA

Electrophoresis systems, Model UEA
Electroeluter, sequencing analysis

International Biotechnologies, Inc. (IBI)
P.O. Box 9558
275 Winchester Avenue
New Haven, CT 06535
(203) 786-5600/(800) 243-2555

Isco
P.O. Box 5347
Lincoln, NE 68505
U.S.A.
(800) 228-4250

Power supplies

John's Scientific Inc.
145 Bentley Avenue, Unit 8
Ottawa, Ontario
K2E 6T7
(613) 727-5630/(800) 268-4410

Assorted equipment, eg., "MSE"
centrifuges, "Lab-line" shakers,
"Perkin - Elmer" spectrophotometers,
etc.

Kontes
Spruce Street,
P.O. Box 729
Vineland, NJ 08360
U.S.A.
(800) 223-7150

Manufacturer of glass products,
scientific instruments, and flowmeters

Lab-line Instruments Inc.
15th and Bloomingdale Avenues
Melrose Park, IL 60160
U.S.A.
(312) 450-2600/(800) 323-0257
Canada

Environmental shakers, ovens,
water baths

Products available from
John's Scientific Inc. and Fisher Scientific

Address	Types of supplies
Mandel Scientific Company Ltd., Hanlon Business Park 2 Admiral Place, R.R. #6 Guelph, Ontario N1H 6J3 (519) 763-2145	Pipetman™ variable volume pipettes
Nalgene Labware Department Nalge Company Division of Sybron Corporation Rochester, NY 14602 U.S.A. (716) 586-8800 Canada Products available from BDH, Canada-wide Scientific, Canlab, Fisher Scientific, John's Scientific	Plasticware, eg., radiation shields, filterware
New Brunswick Scientific Co., Inc. P.O. Box 986 44 Talmadge Road Edison, NJ 08818 U.S.A.	Environmental incubators and shakers
Nucleopore Corporation 7035 Commerce Circle Pleasanton, CA 9456-3294 U.S.A. Canada Products available from Ingram and Bell Scientific 20 Bond Avenue Don Mills, Ontario M3B 1L9 (416) 443-9647	Filtration products
Oxford Laboratories 289 Blakie Rd. London, Ontario N6L 1B8 (519) 652-2400/(800) 265-3496	Suppliers of products from Haake, Lab-line, Falcon, Corning, Schleicher and Schuell, Difco Laboratories, Whatman, Brinkmann, et. al.
Pall Ultrafine Filtration Corporation Glen Cove, NY 11542 U.S.A. Canada ICN Biomedicals Canada Ltd. 1956, rue Bourdon Montreal, Québec H4M 1X1 (514) 744-4548/(800) 361-9616	Biodyne (nylon membrane filters)

<i>Address</i>	<i>Types of supplies</i>
Pegasus Industrial Specialties Ltd. P.O. Box 319 Agincourt, Ontario M1S 3B9 (416) 298-3141	General and custom-made glassware
Perkin-Elmer, Ltd. 376 Churchill Ave., Suite 209 Ottawa, Ontario K1Z 5C2 (613) 726-1944 USA Perkin-Elmer Corporation Oak Brook Instrument Division 2000 York Road Oak Brook, IL 60521 (312) 887-0770	UV spectrophotometer
Pharmacia (Canada) Inc. 500 Morgan Boulevard Baie D'Urfé, Québec H9X 3V1 (514) 457-6661 / (800) 363-7878 (Ont., Québec, Maritimes) USA Pharmacia Inc. 800 Centennial Avenue Piscataway, NJ 08854 (201) 457-8000	Electrophoresis systems, eg. power supplies, LKB products, DNA synthesis (Gene Assembler Plus)
Polaroid Canada 350 Carlingview Drive Rexdale, Ontario M9W 5G6	Polaroid MP-4 Land Camera
Sargent-Welch Scientific of Canada Limited 285 Garyray Drive Weston, Ontario M9L 1P3 (416) 741-5210 USA Sargent-Welch Scientific Company 7300 North Linder Avenue P.O. Box 1026 Skokie, IL 60077 U.S.A. (312) 677-0600	Assorted equipment

<i>Address</i>	<i>Types of supplies</i>
Sarstedt Canada, Inc. 5655 Bois-franc St. Laurent, Qué. H4S 1B2 (514) 337-6908 <i>USA</i> Sarstedt, Inc., Route 1, P.O. Box 4090 Princeton, NJ 08540 (609) 452-1155/(800) 257-5101	Sterile disposable plasticware, eg., pipettes, centrifuge tubes
Savant Instruments, Inc. 110-103 Bi-County Blvd. Farmingdale, NY 11735 U.S.A. (516) 249-4600 <i>Canada</i> Products available from Emerson Instruments Inc. 2275 Victoria Park Ave., Suite 205 Scarborough, Ontario M1R 1W4 (416) 449-8437	"Speed Vac" evaporator-concentrator, centrifuges, electrophoresis systems
Schleicher and Schuell, Inc. 10 Optical Avenue Keene, NH 03431 U.S.A. (603) 352-3810/(800) 245-4024 <i>Canada</i> Products available from Mandel Scientific Company Ltd. 143 Dennis Street Rockwood, Ontario N0B 2K0 (519) 856-9566/(800) 265-8348	Filters, "Elutip" minicolumns, "dot-blot" systems, filtration devices
Sigma Chemical Company P.O. Box 14508 St. Louis, MO 63178	Sequencing pipette, exposure cassettes, acrylic shields and containers
Terochem 2740-A Slough St. Mississauga, Ontario L4T 1G3 (416) 673-0705/(800) 268-4944	Assorted equipment, eg., "Camag" transilluminator; Whatman, Socorex, Ika, Tomy, and Hughes and Hughes products
Transcat P.O. Box 456, Station "A" Rexdale, Ontario M9W 5L4 (800) 387-6878	Assorted equipment

Address**Types of supplies**

Whatman, Inc.
9 Bridewell Place
Clifton, NJ 07014
U.S.A.

Canada

Products available from
Canlab, Fisher Scientific, John's Scientific,
Mandel Scientific, and Terochem

Xymotech Biosystems Inc.
8180 Devonshire, Suite No. 8
Montreal, Qué.
H4P 2K3
(514) 738-1386/(416) 283-2977

Paper filters

Distributors of Schleicher and Schuell
products; eg., pre-cast gel systems,
transfer systems, filtration systems